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UNIVERSITY OF ALBERTA

METABOLITES OF *PHOMA ETHERIDGEI*

BY

LUIS DIEGO JIMENEZ

A THESIS
SUBMTTED TO THE FACULTY OF GRADUATE STUDIES AND
REASEARCH IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER IN SCIENCE

DEPARTMENT OF CHEMISTRY

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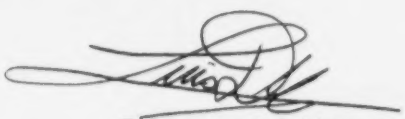

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METABOLITES OF *PHOMA ETHERIDGEI*

submitted by Luis Diego Jimenez in partial fulfillment of the requirements for the degree of Master of Science in Chemistry.

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Date: January 17, 1994.

To my parents and sisters

ABSTRACT

Aspen (*Populus tremuloides* Michx.) and balsam poplar (*Populus balsamifera* L.) are major components of the forest resources in the prairie provinces and northeastern British Columbia. These two species are now recognized as commercially important. They are expected to play a central role in the forest industry in the prairie provinces during the 21st century.

Decay and stain caused by various fungi have been identified as the two most important factors limiting the utilization of aspen. More than 250 species of fungi are known to be associated with decay of North American aspen, and many other non-decay fungi have been isolated from decayed, stained and clear aspen wood. The most common and most important cause of aspen trunk rot in Alberta is *Phellinus tremulae* (Bondartsev) Bondartsev and Borisov.

Trees bearing a black gall are resistant to attack by *P. tremulae*. Therefore, this black gall has become the target of increased biological and chemical research during the last five years.

Isolation of fungi on the surface of black galls from aspen trunks collected in various areas of Alberta has resulted in the isolation of a new fungus, *Phoma etheridgei* sp.nov. Hutchison and Hiratsuka (Can. For. Serv.; North. For. Res. Cent., unpublished results) which shows antagonistic activity against *P. tremulae*. In this work, this activity was found to be due to metabolites of *P. etheridgei* isolated from liquid cultures. The metabolites produced by *P. etheridgei* were examined. Bioassay directed separation of the metabolites led to the isolation of phomalone, the compound responsible for the activity against *P. tremulae*. The isolation, structure determination, bioactivity, and biosynthesis of this new natural product are presented herein.

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I am pleased to express my most sincere gratitude to Prof. W. A. Ayer for his guidance and support during my years as a graduate student.

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ABBREVIATIONS

APT	Attached Proton Test
CzM	Czapeck's Medium
HPLC	High Performance Liquid Chromatography
HPMPLC	High Performance Medium Pressure Liquid Chromatography
INAPT	Insensive Nuclei Assigned by Polarization Transfer
MM	Malt Medium
MEA	Malt Extract Agar
NOE	Nuclear Overhauser Enhancement
NOF	Northern Forest Research Centre
NP-SI 60	Normal Phase-Silica Gel 60
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth

PDYA	Potato Dextrose Yeast Agar
PDYB	Potato Dextrose Yeast Broth
PYEA	Phytone Yeast Extract Agar
PYEM	Phytone Pectone Yeast Extract Medium
RP-18	Reverse Phase C-18
UAMH	University of Alberta Microfungus Collection

I. INTRODUCTION

Aspen (*Populus tremuloides* Michx.) and balsam poplar (*Populus balsamifera* L.) are major components of the forest resource in the prairie provinces and northeastern British Columbia. These two species are now recognized as commercially important. They are expected to play a central role in the forest industry in the prairie provinces during the twenty-first century.

Decay and stain caused by various fungi have been identified as the two most important factors limiting the utilization of aspen.[1,2] More than 250 species of fungi are known to be associated with decay of North American aspen,[3] and many other non-decay fungi have been isolated from decayed, stained and clear aspen wood.[4-7] The most important cause of aspen trunk rot in Alberta is *Phellinus tremulae* (Bondartsev) Bondartsev and Borisov.[8-12] Thomas[13] and coworkers estimated that 38.6% of trunk decay volume in Alberta is caused by this fungus. Basham[14] reported that 63.2 % of 1754 trees on 47 plots in Ontario had trunk rot, and almost 75% of the volume loss was attributed to *P. tremulae*.

Hiratsuka and coworkers[1] have found that aspen trees bearing a certain type of burl or tumor-like structure known as a black galls are resistant to attack by *P. tremulae*. Therefore, this black gall has become the subject of increased biological and chemical research during the last five years.

Isolation of fungi from the surface and interior of black galls from aspen trunks collected in various areas of Alberta has resulted in the isolation of about thirty five species of fungi and several bacteria.[15,16] Among these, a new fungus, *Phoma etheridgei* sp.nov. Hutchison and Hiratsuka (Can. For. Serv.; North. For. Res. Cent., unpublished results) has shown antagonistic activity against *P. tremulae*.[17] This activity has been found to be due to metabolites of *P. etheridgei* described herein.

I. A. ASPEN

I. A. 1. Taxonomy

Aspen, the most widely distributed tree in North America, has a variety of regionally distinct common names: abele poplar, aspen poplar, white poplar, smooth-barked poplar, popple, asp, quaking asp, quaking aspen, and trembling aspen.[18] There is a marked variability in aspen's external appearance due to its predisposition to hybridize, both naturally and through controlled crossing. As a consequence taxonomic classification is not an easy task. The following section briefly describes the generally accepted taxonomic classification of the genus.

The genus *Populus* belongs to the family *Salicaceae* which forms part of the order *Salicaceae* of the group *Amentiflorae*, characterized by unisexual flowers with the perianth absent or insignificant. The *Amentiflorae* falls within the subclass *Monochlamydae* of the class *Dicotyledonae*, subdivision *Angiospermae*, division *Phamerogamae*.[19]

The trees belonging to the family *Salicaceae* present the following features: a) many are reproduced primarily by vegetative processes and not from seed, although aspen is an exception, b) they are dioecious, c) hybridization is frequent between trees of different types and from complementary sexes.[19] Representative species of the genus *Populus* that occur in Canada are listed in table 1.[20-22]

I. A. 2. Geographic distribution

Aspen grows on a variety of soils, but it thrives best on well drained and loamy soils with high lime content.[23] Trembling aspen is a pioneer species on sites disturbed by logging, fire, or other natural disruptions and is highly intolerant of shade.

Table 1. *Populus species* that occur in Canada

Common names	Current botanical name
Trembling aspen, quaking aspen, aspen	*P. tremuloides* Michx.
Large-toothed aspen, largetooth aspen, bigtooth aspen	*P. grandidentata* Michx.
Balsam poplar, hackmatack taccamahac, balm of Gilead	*P. balsamifera* L.ssp. *balsamifera* Brayshaw and *P. balsamifera* L. var. *subcordata* Hylander
Black cottonwood, balsam cottonwood	P. balsamifera L. ssp. *trichocarpa* (Torr. & Gray) Brayshaw
Narrow-leaf cottonwood, willow-leaved cottonwood	*P. angustifolia* James
Eastern cottonwood	*P. deltoides* Marsh
Plains cottonwood	*P. deltoides* Marsh var. *occidentalis* Rydb.

The shallow and wide spreading root system produces abundant sucker regrowth, especially if the stand is logged or killed by fire. Clones resulting from suckering can vary in size from one tree to many thousands, and their growth is very rapid during the first few years. Aspen, in contrast to other species of the *Salicaceae* family, cannot be propagated by cutting, but root cuttings will sprout.[23,24] Schier[25,26] reported general characteristics of physiology of this process. Sexual reproduction of aspen is abundant, and the light cottony seed is carried long distances by the wind. Trembling aspen

grows rapidly during the first 20 years and generally it reaches maturity after 30 to 40 years.[27]

Aspen and balsam poplar are widely distributed in North America (Figure 1).[28,29] The commercial range of aspen-balsam poplar in the Prairie provinces and northeastern British Columbia is illustrated in Figure 2.[30]

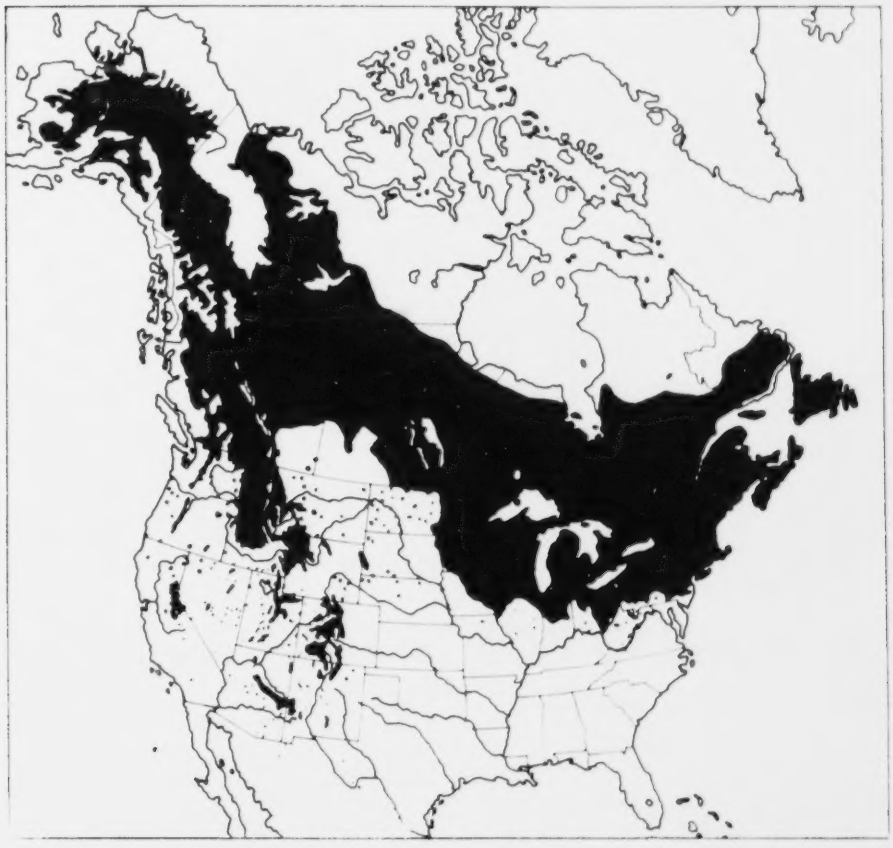


Figure 1. Natural distribution of aspen in North America (adapted from Peterson, E.; Peterson, N. "Ecology, management, and use of aspen and balsam poplar in the Prairie Provinces, Canada", Can. For. Serv.; North. For. Res. Cent., Edmonton, Alberta, 1992, Special report 1, p.12.)

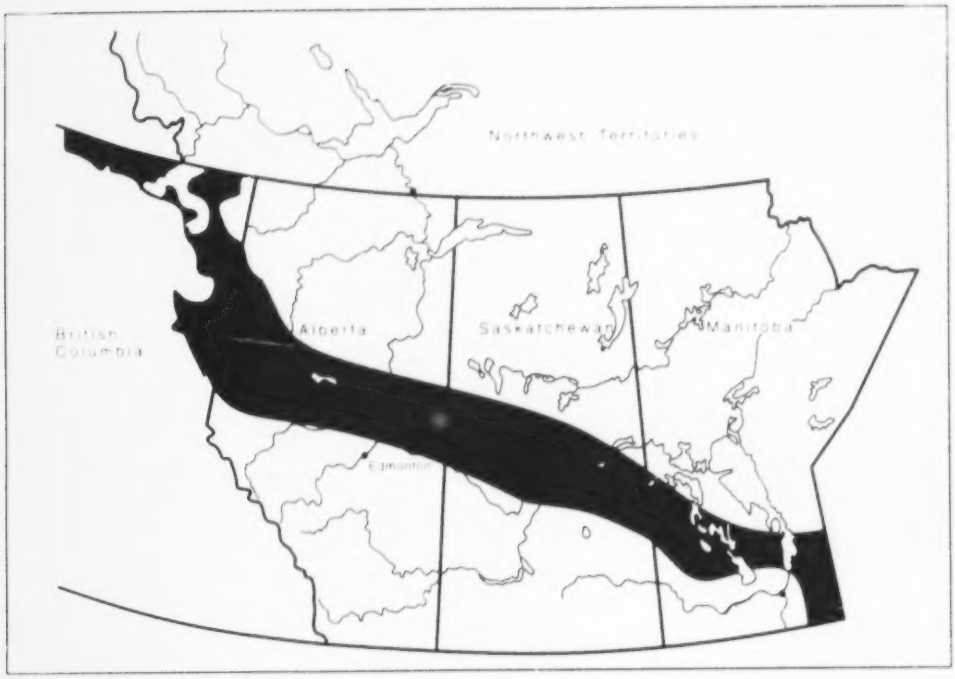


Figure 2. Commercial zone of aspen-balsam poplar in the Prairie Provinces and northeastern British Columbia (adapted from Peterson, E.; Peterson, N. "Ecology, management, and use of aspen and balsam poplar in the Prairie Provinces, Canada", Can. For. Serv.; North. For. Res. Cent., Edmonton, Alberta, 1992, Special report 1, p,1.)

I. A. 3. Economic importance

In Canada, aspen represents 54% of net merchantable hardwood timber and 11% of the entire Canadian net timber resources.[31] In Alberta, aspen comprises about 40% of the total forest resources,[32,33] where the annual aspen-poplar harvest consists of 97% aspen, 2% balsam poplar and 1% black cottonwood (Table 2).[34]

Table 2. Industries in Alberta using aspen-poplar (1987-88)

Industry	No. Plants	Aspen-poplar log input m^3	Aspen-poplar log input %
OSB mills	3	1083133	75.4
Pulp and paper mills	2	220632	15.4
Sawmill-planing mill complexes	117	58155	4.0
Firewood producers	N/A[a]	41500	2.9
Pallet mill	2	31300	2.2
Container mill	1	N/A[a]	
Furniture mills	2	430	
Cattle feed pelleting mill	1	900	
Total	128	1436050	99.9

[a] N/A= not available

The use and economic importance of aspen increased dramatically in the late 1980s (Figure 3).[34] Within Alberta, aspen is currently used as raw material for a broad range of products. Although the use of aspen for pulp is relatively limited, a considerable amount is used in the manufacture of Oriented Strand Board (OSB, a type of particleboard where strands are bonded together with synthetic resins or binders under heat and pressure).[34] In northern British Columbia, aspen is used to make chopsticks and popsicle sticks.

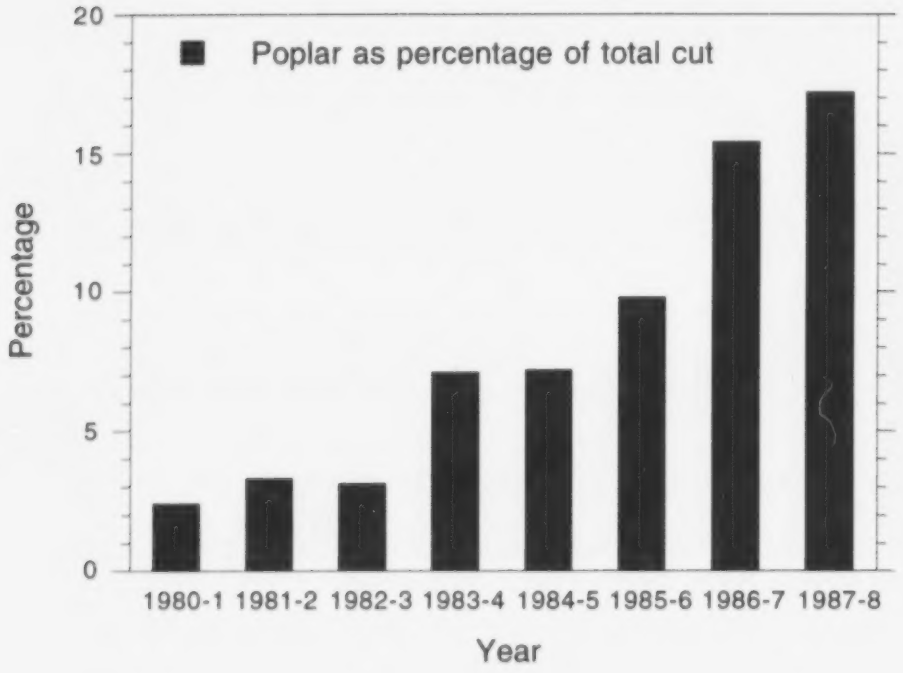


Figure 3. Annual aspen-poplar harvest in Alberta as a percentage of total volume cut. (adapted from Peterson, E.; Peterson, N. "Ecology, management, and use of aspen and balsam poplar in the Prairie Provinces, Canada", Can. For. Serv.; North. For. Res. Cent., Edmonton, Alberta, 1992, Special report 1, p,161.)

I. A. 4. *Phellinus tremulae* decay in aspen and balsam poplar

Basham[8] reports that *P. tremulae* invades the aspen tree only after attack by other fungi which do not cause decay of the wood, but alter it in some way that makes it possible for *P. tremulae* to establish itself. Shigo[35,36] has found that discoloration around rot columns caused by *P. tremulae* is associated with bacteria and non-hymenomycetous fungi.

Aspen stem decay is characterized by a black line that surrounds or occurs within decayed areas. Hoof-shaped conks are characteristic external indicators of *P. tremulae* . The rot caused by *P. tremulae* is white, spongy, and soft. The fungus produces a long decay column which begins about two meters above ground level and that continues throughout most of the main stem.[1]

I. A. 5. Black galls on aspen

The black galls are described as rough oval spots and tissured bands of grayish-black, corky bark that often extends all or part way around the trunk or branches.[37] Crane[38] has done an anatomical and histological study of normal and gall wood and bark to find clues to the cause of these black galls and their effect on aspen.

Results of extensive field surveys conducted in Hinton, Whitecourt and Elk Island National Park near Edmonton during the spring and summer of 1992 indicated that the incidence of decay by *P. tremulae* is significantly less in trees with black galls compared to trees not bearing the galls.[39]

Although the cause of black galls is still not well defined, recent work by Crane[38] has implicated mites, at least in some cases. Several bacteria belonging to the genera *Pseudomonas* and *Agrobacterium* and about thirty five fungi have been isolated from black gall tissues.[15,16] Among these two fungi, *Hyphozyma lignicola* sp. nov. Hutchison and Sigler (Can. For. Serv.; North. For. Res. Cent., unpublished results) and *P. etheridgei* have shown antagonistic activity toward *P. tremulae in vitro.* (Hutchison, L.J.; Chakravarty, P.; Hiratsuka, Y. Can. For. Serv.; North. For. Res. Cent., unpublished results). Many of the above thirty five fungi have been inoculated into young aspen seddlings in an attempt to identify the organism(s) causing the galls. However, no conclusive results have been attained.

I. B. *PHOMA* SPECIES

I. B. 1. Taxonomy

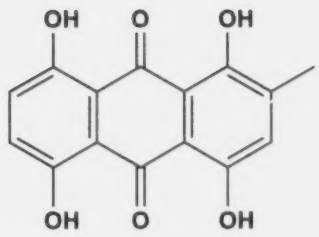
Phoma is the largest and most widely distributed genus of the form order *Sphaeropsidales*.[40] More than 2000 species have been described in the genus. While most are saprophytic on dead or dying plant material, some of them parasitize host plants, including many important agricultural crops.

Hutchison, Chakravarty and Hiratsuka have isolated *P. etheridgei* [17] from the bark of black galls of trembling aspen. Morphological, physiological and molecular studies on this and other isolates confirmed that it corresponds to a new *Phoma* species. A formal taxonomic description of *P. etheridgei* is provided by Hutchison and Hiratsuka (Can. For. Serv.; North. For. Res. Cent., unpublished results).

An isolate of this new *Phoma* species has been deposited in the Fungus Collection of the Northern Forestry Center, Forestry Canada (NOF 1610). In addition, *P. etheridgei* is deposited at the University of Alberta Microfungus Collection (UAMH 7003), and the Canadian Collection of Fungus Cultures in Ottawa.

I. B. 2. Metabolites of *Phoma species*

The genus *Phoma* produces a wide range of natural products. Among these metabolites are: cynodontin (**1**), helminthosporin (**2**),[41-43] phomazarine (**3**),[44] epoxydone or phyllosinol (**4**),[45,46] gentisyl alcohol (**5**), chlorogentisyl alcohol (**6**), gentisyl acetal (**7**), toluhydroquinone (**8**),[47] phomalactone (**9**),[48] phomin or cytochalasin B (**10**)[49-53], and 5-dehydrophomin or cytochalasin A (**11**).[52,53]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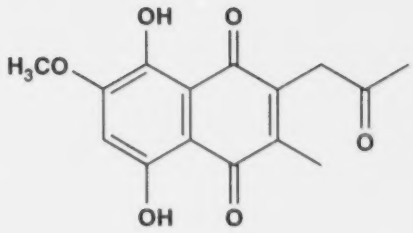
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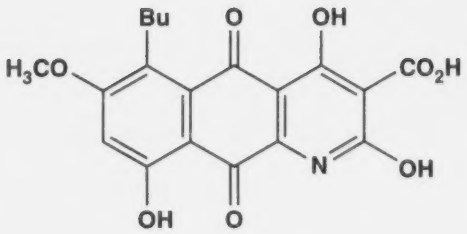
O
OH
O

2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CO2H
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OH
OH
O

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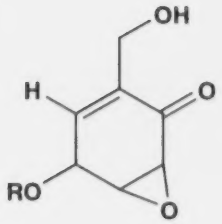
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RO
O

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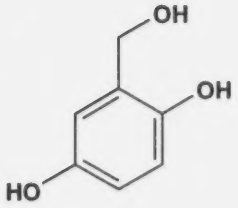
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HO

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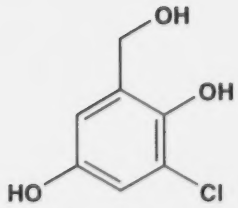
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OH
HO
Cl

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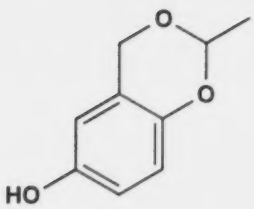

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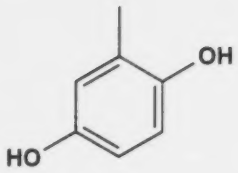

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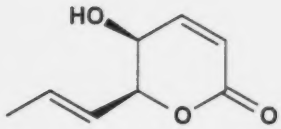

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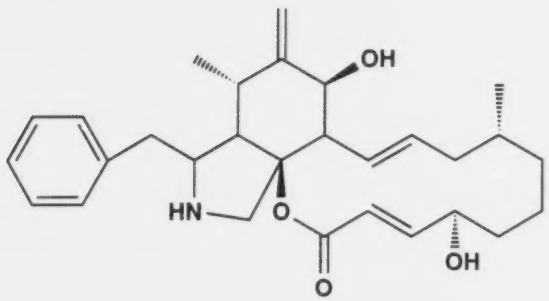

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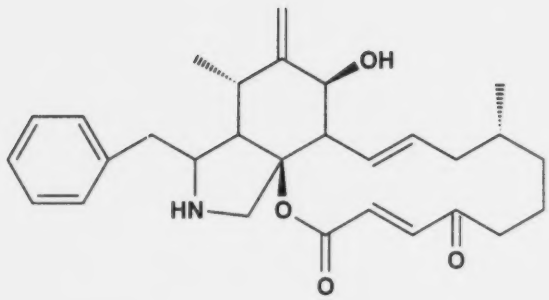
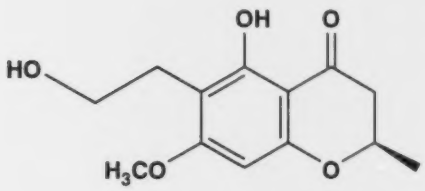


11

Due to the cytotoxic activity shown by cytochalasin A and B,[49-53] in the early 60s, *Phoma* became an important genus in the screening program of mycotoxin-producing fungi.

In 1972, McGahren and coworkers[54] reported the isolation of 2-methylchromanones, LL-D253α (**12**), LLD-253β (**13**) and LLD-253γ (**14**) from *Phoma pigmentivora* Boerema.



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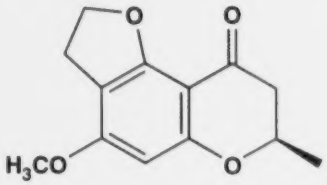

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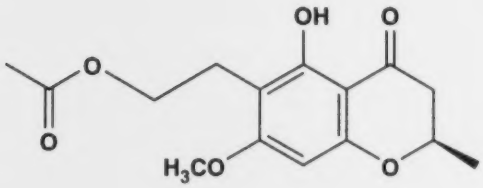


14

Kurata and coworkers[55] isolated LL-D253α from a *Phoma* strain in 1973. This Japanese group reported cytotoxic activity against cultured HeLa cells (cancer cells) and mice. However, these studies were not conclusive and no further studies are reported.

Hutchison and coworkers[17] found antagonism between *P. tremulae* and *P. etheridgei in vitro*. *P. etheridgei* produced an inhibition zone around a *P. tremulae* colony after two weeks on malt extract agar and carrot agar medium in Petri dishes. Moreover, Hutchison and coworkers[17] noted similar antagonism on popsicle and aspen sticks. In this thesis, the metabolites produced by *P. etheridgei* which are responsible for this activity are reported.

II. RESULTS AND DISCUSSION

Aspen trees bearing a certain type of burl or tumor-like structure known as black galls are resistant to attack by *P. tremulae*. Therefore, this black gall has become the subject of increased biological and chemical research during the last five years. Isolation of fungi from the surface and interior of black galls from aspen trunks has resulted in the isolation of about thirty five species of fungi and several bacteria. Among these a new fungus, *P. etheridgei*, has shown antagonistic activity against *P. tremulae*.

The chemical research on *P. etheridgei* investigated in this work involved four points: a) evaluation of inocula and liquid media for reproducible cultures, b) isolation and structure elucidation of compounds active against *P. tremulae*, c) study of the biosynthesis of the compound responsible for activity, and d) confirmatory bioassays.

II. A. EVALUATION OF INOCULA AND LIQUID MEDIA FOR GROWING CULTURES

In order to obtain a reproducible batch of liquid cultures, it was necessary to establish optimum culture conditions for *P. etheridgei*. Investigation on the behavior of *P. etheridgei* under different culture conditions was the first objective in this project.

Since *P. etheridgei* reproduces by asexual means, a brief description about its physiology and growth is presented here. Like most fungi, *P. etheridgei* is characteristically filamentous, or thread like. The individual threads are called hyphae which usually branch in order to produce mycelium.[40,56] The individual hyphae are surrounded by rigid cell walls which have apical growth similar to most other fungi. *P. etheridgei*, in culture, reproduces by asexual means, and produces spores as a final product. These bodies which are able to develop an individual with the same genetic pattern are called conidia and the fruiting bodies that produce conidia are called pycnidia.

In this asexual reproduction of *P. etheridgei*, in cultures, mycelium or conidia can be used as inoculum for a new colony. The effects of culture media, temperature and light on radial growth and pycnidium production were investigated *in vitro*. Four culture media: potato dextrose agar (PDA), potato dextrose yeast agar (PDYA), malt extract agar (MEA) and phytone yeast extract agar (PYEA) were tested. Twenty ml of each medium (sterilized) was dispensed on each of four replicate 90 mm diameter Petri dishes and inoculated with 7 mm diameter MEA plugs of 12-days old colonies from young actively growing peripheral mycelium. Inoculated plates (16 in total) were summited to different experimental conditions. Radial growth was measured 8 and 14 days after inoculation by dividing by two the mean of two diameter readings of each plate taken at right angles to each other. The extent of pycnidium production in these cultures was also determined at 8 days after inoculation. These estimates were based on the pycnidia counting for each Petri dish. These pycnidia were determined using the graduated surface of the hemacytometer with the aid of a microspore equipped with a reichert wien.

There was some effect of culture media on the radial growth and pycnidium production. MEA and PYEA Petri dishes corresponded with high pycnidium production (high counting for pycnidia and low radial growth), while on other media (PDA, PDYA) were generally associated with mycelium production (high radial growth and low counting pycnidia).

The effect of temperature on growth and pycnidium production were tested in two media: MEA and PYEA. After inoculation, four replicate Petri dishes for each media (8 in total) were incubated at 5 °C, 10 °C, 15 °C, 20 °C, 25-28 °C (room temperature), 30 °C, and 35 °C. Plates of each medium were randomly arranged on one incubator while those for the room temperature treatment were also randomly arranged on a laboratory bench. Temperatures 5 °C, 10 °C and 15 °C were tested first, followed by 15 °C, 20 °C, 25-28 °C (room temperature), and 30 °C, with 35 °C coming last. Radial growth measurements of colonies and extent of pycnidium production were determined as described above, 8 and 14 days, respectively after inoculation.

The radial growth increased with the temperature, for a maximum observed at 30 °C. Higher temperatures showed a deflexion toward lower radial growth. Cultures on MEA and PYEA showed the same temperature effect.

The effect of temperature on pycnidium production showed that temperatures of 5 °C, 10 °C and 35 °C inhibited pycnidium formation completely. There was no evidence for the medium effect. MEA and PYEA showed maximum pycnidium production at room temperature. Temperatures of 15 °C and 20 °C showed slow pycnidium production (low pycnidia counting) relative to that at room temperature.

The effect of light on pycnidium production was evaluated on four media: MEA, PYEA, PDA and PDYA. After inoculation, four replicate Petri dishes for each media (16 in total) were incubated for 8-14 days under four different light conditions: 1) continuous white fluorescent light from a 40 W 120 cm-length tube, 40 cm above the Petri dishes; 2) alternate periods of darkness and light, twelve hours each, 3) continuous darkness, and 4) continuous near-ultraviolet radiation ($\lambda < 360$ nm) from a 40 W 60 cm-length tube 40 cm above the Petri dishes. Treatment 2 was arranged on a laboratory bench, and incubated at room temperature (25-28 °C) with 12h white fluorescent light (1.2 m above the Petri dishes) and 12 h darkness. The other three treatments were randomly arranged under each light condition at 25 °C. Pycnidium production was measured roughly six and fourteen days after inoculation (pycnidia counting using the hemacytometer). An initial analysis showed a remarkable interaction between *P. etheridgei* and the media. In MEA and PYEA, pycnidia were produced under any of the above treatments of light. In PDA and PDYA, mycelium was produced under any light treatment, but no picnidia production was observed. In some cases pycnidia appeared after nine weeks under condition 2 (twelve hours of darkness/light). However, inoculation of these pycnidia in a new Petri dish did not produce new colonies of *P. etheridgei*. Under continuous darkness (condition 3), pycnidia production was observed on PYEA and MEA media. On PDA and PDYA, *P. etheridgei* did not sporulate, and produced a mixture of clear and dark mycelium. Under treatment 4 (near uv-light), there was a significantly higher pycnidium production on MEA and PYEA media during the first week of growing.

From the results above, it was concluded that media supporting the best mycelial growth(PDA and PDYA) were not necessarily those which enhanced formation of pycnidia. *P. etheridgei* produce pycnidia in MEA and PYEA in the range 25-30 °C under any light conditions. The stimulatory effect of near-ultraviolet radiation for pycnidium production observed in these studies has similarly been reported in other *Phoma* sp., for instance, *Phoma medicaginis* var. *pinodella* L. K. Jones, *Phoma trifolii* Johnsos and Valleau.[57,58] In these studies, it was found that near uv-light increase the pycnidia production during the first week.

The production of active metabolites seems to be very dependent on the inoculum used for the liquid culture. The use of mycelium as inoculum produced some clear and some dark broths among several cultures in the same batch. For instance, using a plug (7 mm diameter) cut from a clear PDA plate the culture produced only clear broth with a weak activity against *P. tremulae*. Using a plug from a dark PDA plate, a dark broth with no activity against the growing of *P. tremulae* was obtained. Mycelium of either early or late stage (see experimental section) produced an heterogeneous batch where metabolites from clear broth were different from those from dark broth.

Spore suspension was found to be the best inoculum for the liquid cultures. *P. etheridgei* produce pycnidia in PEA and MEA petri dishes within a reasonable period of time (two to four weeks).

After having definided the inoculum, five liquid media were tested: malt medium (MM), phytone peptone yeast extract medium (PYEM), potato dextrose yeast broth (PDYB), potate dextrose broth (PDB), and Czapeck's medium (CzM).

P. etheridgei produced active metabolites when grown in malt medium (MM) or phytone peptone yeast extract medium (PYEM) in shake cultures, with variable production of mycelium. In PDYB an active broth was obtained, but its activity was lower than that of MM or PYEM. In CzM the broth did not show activity against *P. tremulae*, although it produced more mycelium in shake

culture as compared to the others. In PDB no activity was observed, and the lowest amount of mycelium was obtained. Different light treatment did not seem to have an effect on mycelium production or net activity in shake cultures. However, additional metabolites were obtained under condition 2 (twelve hours of darkness/light) in MM.

In order to improve the production of active compounds, the five liquid media (MM, PYEM, PDYB, PDB and CzM) were evaluated after growing *P. etheridgei* in still cultures. These liquid cultures were randomly arranged on a laboratory bench, and incubated at room temperature (25-28 °C) with 12 h white fluorescent light and 12 h darkness. In these conditions, the five liquid media led to the formation of a comparable quantity of mycelium. However, only on MM a weak activity was found.

From the results described above, it was concluded that still cultures produce a higher amount of mycelium. Nevertheless, production of active metabolites did not seem to be associated with the amount of mycelium being produced. All the active metabolites were found in the broth of the liquid cultures which has been obtained in shake cultures.

Since cultures in MM and PYE broths showed activity against the growth of *P. tremulae*, these media were selected for the production of active metabolites. It was found that the best conditions are: shake cultures at room temperature temperature (25-28 °C) with 12 h white fluorescent light and 12 h darkness during twelve to sixteen days as growing time.

II. B. ISOLATION AND STRUCTURE ELUCIDATION OF COMPOUNDS ACTIVE AGAINST *P. tremulae*

Bioassay against growth of *P. tremulae* on MEA was used to direct the separation of active compounds. Of the five isolation procedures, extraction of the broth or the HP-20 resin (see experimental section for details) from MM or PYEM medium, afforded crude extracts with a positive bioassay. However, as mentioned above, metabolites from MM-broth appeared to be different from

those obtained from PYEM-broth under light condition 2. Phomalone, the major compound responsible for the activity, was isolated from both media, but MM medium provided additional metabolites whose structure elucidation will also be discussed.

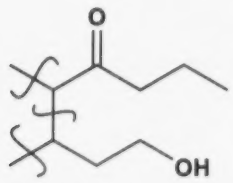
II. B. 1. Structure Elucidation of the Active Compound Phomalone

The ir spectrum of phomalone shows absorptions at 3200 cm^{-1} (OH, H-bonded hydroxyl group), 1618 cm^{-1} (ketone C=O), 1448 cm^{-1} and 1427 cm^{-1} (Ar-H). The low frequency of the carbonyl group suggests an carbonyl group *ortho* to a phenolic moiety.

High resolution electron impact mass spectrum (hreims) data shows the molecular ion at m/z 254, corresponding to a molecular formula $C_{13}H_{18}O_5$. The appearance of a strong peak at m/z 223 corresponding to the loss of CH_2OH from the molecular ion suggests the presence of a primary alcohol. In addition, the compound gave a positive test for phenols with ferric chloride.

The ^{1}H-nmr spectral data reveals the presence of four different methylene groups. Two methylene groups appear considerably downfield (δ 2.74, δ 2.80), suggesting that they are linked to unsaturated carbon. Another methylene group appears further downfield (δ 3.70) suggesting the presence of a carbon bearing an oxygen atom. A downfield three proton singlet (δ3.85) reveals the presence of a methoxy group. The remaining hydrogens appear as a singlet at δ 6.01, a singlet at δ 14.5, an apparent sextet at δ 1.66 (J=7.0 Hz, J'=7.0 Hz), and a triplet at δ 0.95 (J=7.0 Hz). The ^{13}C-nmr spectrum shows signals for the groups mentioned above: a downfield carbon at δ 206.4, suggesting a carbonyl group, five aromatic sp^2 carbons between δ 165.0 and δ 105.0, a sp^2 carbon as a doublet at δ 92.1, a sp^3 carbon as a triplet at δ 62.9, suggesting a carbon bearing an oxygen atom, a sp^3 carbon as a quartet at δ 55.9 implying a methoxy group, sp^3 carbons at δ 16.9 as a triplet and δ 14.2 as a quartet suggesting an ethyl group.

Homodecoupling experiments (Table 3) established a partial carbon skeleton allowing the assignment of structural unit **15**.



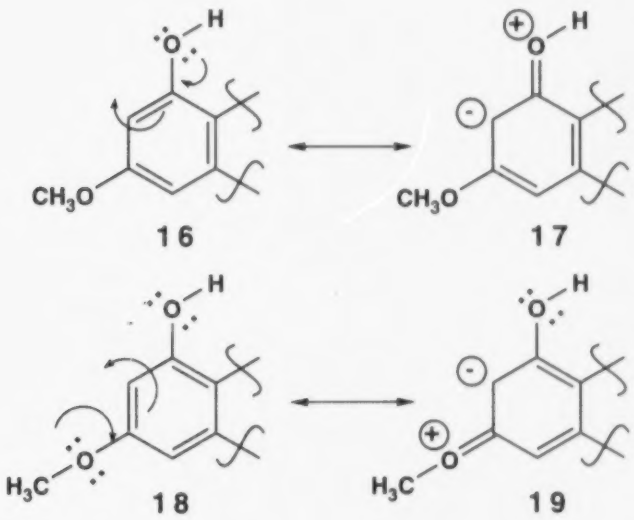
15

Table 3. Homodecoupling experiments on phomalone (Acetone-d_6, 400 MHz)

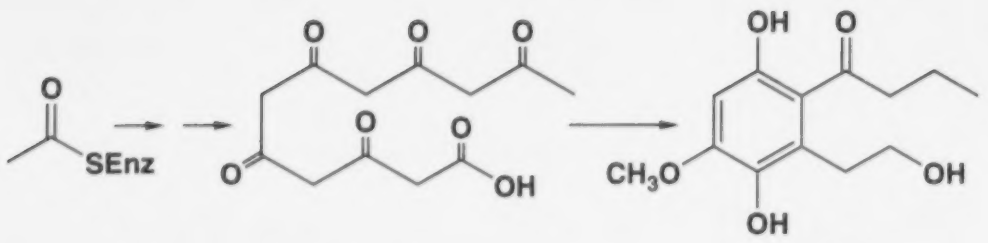
Saturated signal δ	Simplified signal δ	Observation
0.95	1.66	Sextet-triplet (methylene group)
1.66	0.95	triplet-singlet (methyl group)
	2.80	triplet-singlet (methylene group)
2.80	1.66	sextet-quartet (methylene group)
2.74	3.70	triplet-singlet (methylene group)
3.70	2.74	triplet-singlet (methylene group)

For the aromatic ring, the ^{1}H-nmr spectrum shows a singlet at high field (δ 6.01) suggesting an aromatic hydrogen in a phenolic moiety. Higher electronic density at the ortho position is consistent with resonance structures **17** and **19** (Scheme 1). A unsubstituted aromatic carbon flanked by two oxygenated carbons is confirmed by the presence of this sp^2 carbon at high field (δ 92.0) in the ^{13}C-nmr spectrum.

A carbonyl group *ortho* to a phenolic moiety was indicated by a singlet at low field (δ 14.5) in the ^{1}H-nmr spectrum. The relative position of the methoxy group and the hydrogen on the aromatic ring was determined by a NOE experiment.[59] The hydrogen at δ 6.01 exhibited 2.96% NOE enhancement upon irradiation at the signal at δ 3.85, while the methoxy group at δ 3.85 showed a 3.65% NOE upon irradiation of the signal at δ 6.01. This indicates the methoxy group to be located *ortho* to the aromatic proton. At this stage it was possible to define the functional groups present: an aromatic ring, a carbonyl group, a primary alcohol and a methoxy group. A tentative structure **20** is shown for phomalone. This structure may be derived from a hexaketide precursor as shown in Scheme 2.



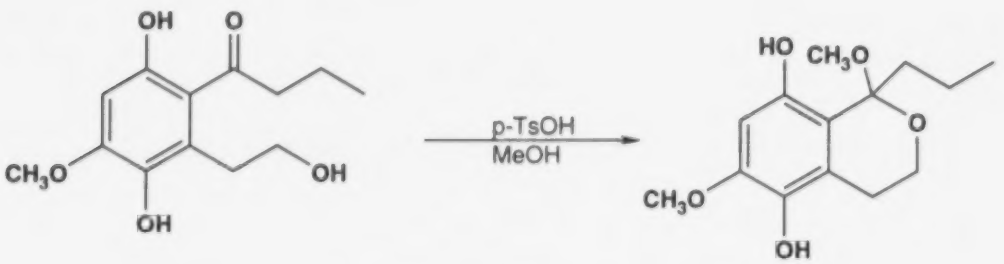
Scheme 1



20

Scheme 2

In order to confirm the position of the hydroxyethyl group relative to the side chain bearing the carbonyl group, a cyclization reaction, using either *p*-TsOH or dry HCl in methanol, was attempted. Scheme 3 shows the expected product.

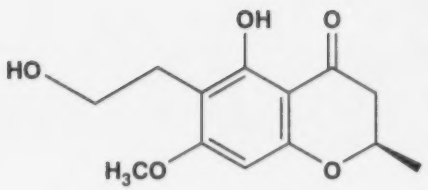


20 21

Scheme 3

Since this cyclization reaction did not occur, this arrangement of the functional groups in the aromatic ring was called into question.

In 1992, Simpson and coworkers[60] reported the structural revision and synthesis of LL-D253α, a chromanone metabolite of *P. pigmentivora*. According to Simpson, LL-D253α possesses structure **22** and not **12** as McGahren[54] and Kurata[55] had reported previously.



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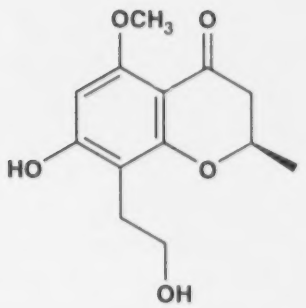
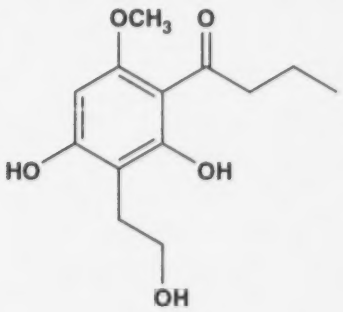


22

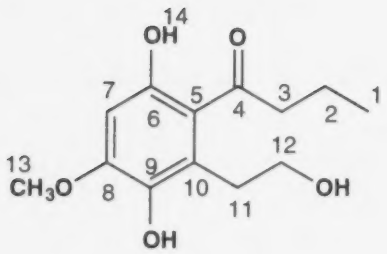
The carbon skeleton for LL-D253α suggests **23** as a possible structure for phomalone.



23

Structures **20** and **23** are both consistent with the spectroscopic data discussed so far for phomalone. In fact, there is not enough evidence for the assignment of the position of the methoxy, hydroxyethyl, and the side chain bearing the carbonyl group.

Insensitive Nuclei Assigned by Polarization Transfer (INAPT)[61-63] experiments provided evidence for the correct assignment of the ^{13}C data, and the connectivity of the functional groups on the aromatic ring. Table 4 summarizes the experimental results. Assignment of the observed signals in the INAPT experiment is described according to the numbering shown in **20** and **23**.



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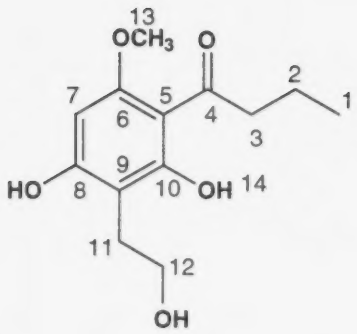


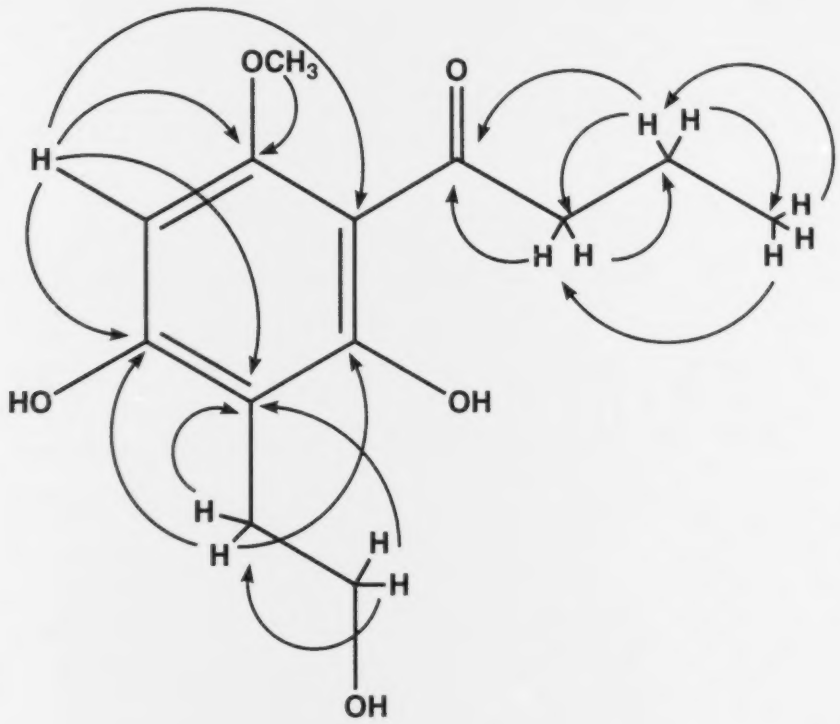
23

Table 4. INAPT on phomalone (DMSO-d_6, 500 MHz)

Saturated signal ^{1}H-nmr (δ)	Observed signal ^{13}C-nmr (δ)	Description
5.09	163.5	oxygenated aromatic carbon
	161.0	oxygenated aromatic carbon
	104.5	quaternary aromatic carbon
	103.8	quaternary aromatic carbon
3.85	161.0	oxygenated aromatic carbon
3.72	104.5	quaternary aromatic carbon
	25.9	secondary aliphatic carbon
2.80	204.6	carbonyl group
	17.8	secondary aliphatic carbon
2.75	164.4	oxygenated aromatic carbon
	163.5	oxygenated aromatic carbon
	104.5	quaternary aromatic carbon
	59.8	secondary aliphatic carbon
1.70	45.2	secondary aliphatic carbon
	13.8	secondary aliphatic carbon
0.98	45.2	secondary aliphatic carbon
	17.8	secondary aliphatic carbon

In both structures, ^{1}H-^{13}C long-range correlation for hydrogens of the methylene group at C11 would show correlation with three aromatic carbon atoms. If structure **20** were correct, hydrogens at C11 would show correlation with two quaternary carbons (C5 and C10), and one oxygenated carbon (C9). In contrast, in structure **23**, hydrogens at C11 would show correlation with two oxygenated carbons (C8 and C10) and one quaternary carbon (C9). The experimental result indicates that hydrogens at C11 (δ 2.75) do show correlation with three aromatic carbons: one quaternary and two oxygenated carbon atoms. This observation suggests that the hydroxyethyl group and the side chain bearing the carbonyl group have a *meta*-relationship on the aromatic ring and that the carbon bearing the hydroxyethyl group is located between two oxygenated carbons. This is not consitent with structure **20**. In addition, ^{1}H-^{13}C long-range correlation results for the aromatic hydrogen (δ 5.09) were consistent with structure **23**. The aromatic hydrogen shows correlation with four aromatic carbons: two quaternary and two oxygenated carbon atoms. In structure **20** the aromatic hydrogen at C7 would show correlation with one quaternary carbon, and three oxygenated carbons. Therefore, the relative position shown on structure **23** for the hydroxyethyl and the side chain bearing the carbonyl group on the aromatic ring is confirmed. Correlation of the hydrogens of the methoxy group (δ 3.85) allowed the assignment of the aromatic carbon bearing the methoxy group (δ 55.4). Consequently, the second oxygenated aromatic carbon that showed correlation with the aromatic hydrogen had to correspond to the signal at δ 163.5. The assignment of the third oxygenated aromatic carbon was established by the correlation between the hydrogens of the methylene group at C11 (δ 2.75), and it is the carbon producing the signal at δ 164.4. Similarly, the assignment of the quaternary aromatic carbon that shows correlation to the hydrogens at C11 has to be C9 (δ 104.5) and the second carbon of the hydroxyethyl group (C12) has to correspond to the signal at δ 59.8. At this point, assignment of five of the aromatic carbons have been established. Therefore, the last aromatic signal at δ 103.8 is assigned to C5. This assignment is confirmed by the correlation between the aromatic hydrogen and two quaternary aromatic carbons. Since C9 was already assigned to δ 104.5; C5 has to correspond to δ 103.8. The correlation of hydrogens of the methylene groups in the side chain allow the assignment of C4, C3, C2 and

C1. Hydrogens at C3 show correlation with C4 (δ 204.6) and C2 (δ 17.8); hydrogens at C2 show correlation with C3 (δ 45.2) and C1 (δ 13.8), and hydrogens at C1 show correlation with C2 (δ 17.8) and C3 (δ 45.2). Scheme 5 summarizes the long range ^{1}H-^{13}C correlations on phomalone (DMSO-d_6, 500 MHz), and shows the final structure assigned to phomalone.

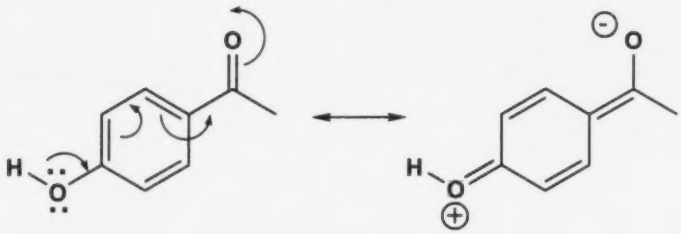


Scheme 5

Structures such as **20** found some support in the ir data. Low frequencies for the carbonyl absorptions (around 1640 cm^{-1}) have been assigned to chelated carbonyl group*ortho* to a phenolic moiety.[64-66] However, the low frequencies of such structures are not due to hydrogen bonding, but to the contribution of the resonance form **25,** which reduces the double bond character of the carbonyl group. A similar resonance form **27** is obtained for *para* substitution. Substitution in the *ortho* or *para* positions produces similar low frequency shifts (ca 50 cm^{-1}), while *meta* substitution produces smaller shifts.[67,68] Multiple

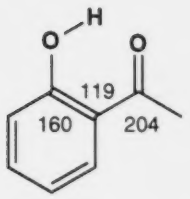
substitution in the aromatic ring, such as in phomalone, has only a small effect (the shifts are not additive). Other evidence for this *ortho* carbonyl group to a phenolic moiety is the higher absorption frequency observed in the ir spectrum for the carbonyl (around 1675 cm^{-1}) after methylation or acetylation. The ir spectrum of phomalone triacetate shows absorption at 1699 cm^{-1} (see section II.B.3) Structure **23** is consistent with these observations.

24 **25**

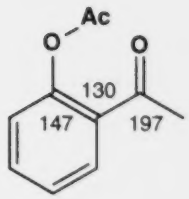


26 **27**

Kobayashi[69] has reported that ^{13}C-nmr data provides evidence for the carbonyl group*ortho* to a phenolic moiety (a so called peri-hydroxy function[69]). It is expected that comparison of the ^{13}C-nmr data for structures like **28** and its acetate (**29**) would show significant changes. After acetylation, the carbonyl and the aromatic carbon bearing the hydroxyl group show lower δ values, while the intermediate carbon moves at higher field. These changes in the ^{13}C chemical shift values of system such as **28** are expected when the chelation of the phenolic moiety is removed, for instance, when 2-hydroxyacetophenone (**28**) is acetylated the ^{13}C resonances at 204, 119 and 160 ppm move to 197, 130 and 147 ppm respectively.

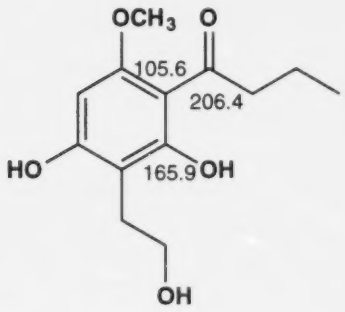


28

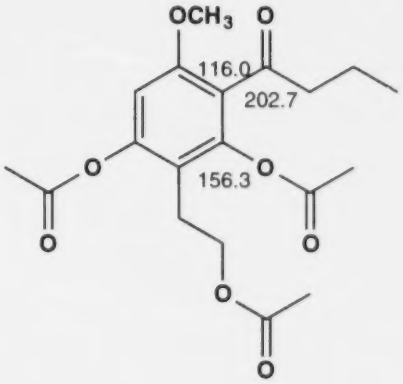


29

Since the same changes were observed in the ^{13}C-nmr data of phomalone (**23**) and the triacetate derivative (**29**), the presence of a chelated phenolic moeity was confirmed. When phomalone is acetylated the ^{13}C resonances at 206.4, 105.6 and 165.9 ppm move to 202.7, 116.0 and 156.3 ppm respectively.



23



30

From the resuts above, 23 shows the final structure for phomalone. Tables 5 and 6 summarize the final nmr assignments for phomalone.

Table 5. ^{1}H-nmr of phomalone (Acetone-d_6, 400 MHz).

Signal δ (m, area, J)	Assignment
0.95 (t, 3H, J=7.0 Hz)	1
1.66 (tq, 2H, J=J'=7.0 Hz)	2
2.74 (t, 2H, J=7.0 Hz)	11
2.80 (t, 2H, J=7.0 Hz)	3
3.70 (t, 2H, J=7.0 Hz)	12
3.85 (s, 3H)	13
6.01 (s, 1H)	7
14.50 (s, 1H)	14

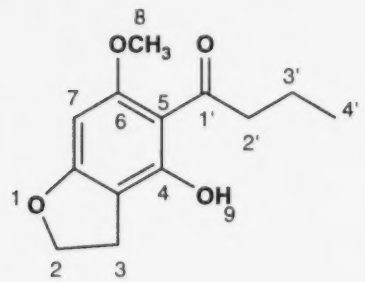
Table 6. ^{13}C-nmr of phomalone (Acetone-d_6, 100.6 MHz)

Signal δ	Assignment
14.2	1
16.9	2
26.3	11
46.6	3
55.9	13
62.9	12
92.1	7
105.6	5
106.9	9
162.7	6
164.3	8
165.9	10
206.4	4

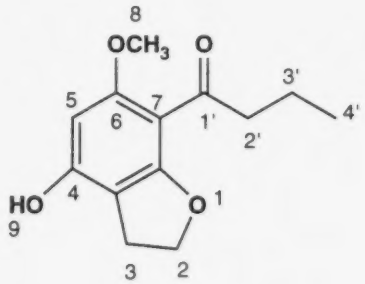
II. B. 2. Phomalone related compounds

II. B. 2. 1. 4-Hydroxy-6-methoxy-5-(1'-oxobutyl)benzo[b]dihydrofuran

The broth of MM-liquid cultures was extracted with ethyl acetate to obtain an organic extract as a viscous-brown oil. Flash chormatography of this oil afforded a white solid which gave a color reaction with ferric chloride. The ir spectrum shows absorption for a hydroxyl group (3200 cm^{-1}) and a low frequency carbonyl absorption (1645 cm^{-1}). Therefore, a carbonyl group *ortho* to a phenolic moiety was considered. The hreims gives a molecular formula $C_{13}H_{16}O_4$ suggesting six unsaturation equivalents. The ^{1}H-nmr spectrum shows signals similar to those of phomalone, but one methylene group was shifted to lower field (δ 4.50 compared with δ 3.70). Thus, the basic carbon skeleton of phomalone seemed to be maintained. The extra unsaturation could then be attributed to a new ring. At this stage, structures **31** and **32** were considered as possibilities.



31



32

The ^{1}H-nmr spectrum shows a singlet at δ 14.50, confirming the presence of the crabonyl group*ortho* to a phenolic moiety. Structure **31** is consistent with this observation, and is assigned to the natural product. Tables 7 and 8 summarize the nmr data.

Table 7. ^{1}H-nmr of 4-hydroxy-6-methoxy-5-(1'-oxobutyl)benzo[b]dihydrofuran ($CDCl_3$, 400 MHz).

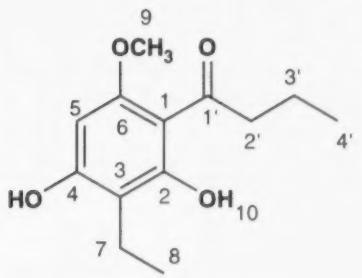
Signal δ (m, area, J)	Assignment
0.98 (t, 3H, J=7.0 Hz)	4'
1.60 (qt, 2H, J=J'=7.0 Hz)	3'
2.80 (t, 2H, J=7.0 Hz)	2'
3.20 t, 2H, J=7.0 Hz	3
3.80 (s, 3H)	8
4.50 (t, 2H, J=7.0 Hz)	2
5.90 (s, 1H)	7
14.50 (s, 1H)	9

Table 8. ^{13}C-nmr of 4-hydroxy-6-methoxy-5-(1'-oxobutyl)benzo[b]dihydrofuran ($CDCl_3$, 100.6 MHz).

Signal δ	Assignment
14.2	4'
18.9	3'
26.4	3
46.6	2'
55.9	8
62.9	2
92.4	7
132.9	5
142.6	3a
162.7	6
164.3	7a
165.9	4
206.4	1'

II. B. 2. 2. 3-Ethyl-2,4-dihydroxy-6-methoxybutyrophenone

Flash chromatography following by prep-TLC of the organic extract afforded another white solid which gave a color reaction with ferric chloride. The ir spectrum shows absorption for a hydroxyl group (3200 cm^{-1}) and a low frequency carbonyl absorption (1618 cm^{-1}). The hreims gives a molecular formula $C_{13}H_{18}O_4$ suggesting five unsaturation equivalents. The ^1H-nmr spectrum shows a similar set of signals to that of phomalone. However, a new methyl group at δ 1.12 was observed as a triplet, and one of the methylene groups was shifted upfield to δ 2.59, compared to δ 2.74 in phomalone. Homodecoupling experiments confirmed that these signals were coupled (J=7.0 Hz). Therefore, it was apparent that the hydroxyethyl group had been replaced by an ethyl group. Structure **33** is consistent with these observations. The nmr data of 3-ethyl-2,4-dihydroxy-6-methoxybutyrophenone are summarized in Tables 9 and 10.



33

Table 9. ^{1}H-nmr of 3-ethyl-2,4-dihydroxy-6-methoxybutyrophenone ($CDCl_3$, 400 MHz).

Signal δ (m, area, J)	Assignment
0.98 (t, 3H, J=7.0 Hz)	4'
1.12 (t, 3H, J=7.0 Hz)	8
1.70 (tq, 2H, J=J'=7.0 Hz)	3'
2.59 (q, 2H, J=7.0 Hz)	7
2.98 (t, 2H, J=7.0 Hz)	2'
3.52 (s, 3H)	9
5.90 (s, 1H)	5
14.35 (s, 1H)	10

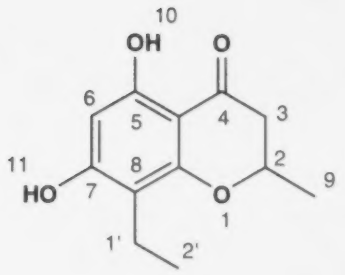
Table 10. ^{13}C-nmr of 3-ethyl-2,4-dihydroxy-6-methoxybutyrophenone ($CDCl_3$, 100.6 MHz).

Signal δ	Assignment
13.40	8
14.0	4'
18.2	3'
22.3	7
46.3	2'
55.4	9
90.1	5
105.7	1
109.5	3
159.6	6
161.5	4
164.9	2
202.3	1'

II. B. 2. 3. 8-Ethyl-5,7-dihydroxy-2-methylchroman-4-one

A third colorless solid was obtained from the organic extract. This also gave a color reaction with ferric chloride. The ir spectrum shows an absorption of a hydroxyl group (3133 cm^{-1}) and a low frequency carbonyl absorption (1631 cm^{-1}). Therefore, a carbonyl group *ortho* to a phenolic moiety was assumed to be present. The ^{1}H-nmr spectrum shows two different methyl groups: a triplet at δ 1.09 (J=7.0 Hz) and a doublet at δ 1.40 (J=7.0 Hz). There are complex signals at δ 2.60 (four hydrogens) and δ 4.50 (one hydrogen). Because of its appearance at low field, the latter suggests a methine group attached to an oxygen atom. The signal at δ 2.60 suggests the presence of two methylene groups attached to an unsaturated system. The remaining signals appear as singlets at δ 6.10, δ 9.50 and δ 12.50. The ^{13}C-nmr spectrum shows signals for the groups mentioned above: seven sp^2 carbons and five sp^3 carbons. A downfield signal at δ 191.7 indicates a carbonyl group, and signals between δ 104.2 and δ 161.5 are consistent with an aromatic ring. One of these signals at δ 90.9 appears as a doublet suggesting an non-substituted aromatic carbon. In addition, its position at high field confirms an aromatic carbon flanked by two oxygenated carbons. This observation is consistent with the presence of a singlet at δ 6.10 in the ^{1}H-nmr. A signal at δ 45.9 in the ^{13}C-nmr spectrum indicates a methylene group linked to carbonyl group. A signal for a sp^3 carbon at δ 73.5 confirms the presence of a carbon bearing an oxygen atom. Signals for sp^3 carbons at δ 22.3, as a triplet, and at δ 13.4, as a quartet, suggest an ethyl group. Another signal for a sp^3 carbon at δ 20.3, as a quartet, confirms the presence of a methyl group.

The appearance of a strong peak at m/z 222 gave a molecular formula of $C_{12}H_{14}O_4$ in the hreims. These results indicate six unsaturation equivalents: four for the aromatic ring, one for the carbonyl group and one for an extra ring. At this stage, it was possible to define the structural units, similar to those in phomalone: an aromatic ring, a carbonyl group, two phenolic groups, an ethyl group and a methine group bearing an oxygen atom. Based on the relative positions of the different groups in phomalone, structure **34** is proposed for this new compound. The nmr data are shown in Tables 11 and 12.



3 4

Table 11. ^{1}H-nmr of 8-ethyl-5,7-dihydroxy-2-methylchroman-4-one (Acetone-d_6, 400 MHz).

Signal δ (m, area, J)	Assignment
1.09 (t, 3H, J=7.0 Hz)	2'
1.40 (d, 3H, J=7.0 Hz)	9
2.60 (m, 4H)	3,1' overlapping
4.50 (m, 1H)	2
6.10 (s, 1H)	6
9.50 (s, 1H)	11
12.50 (s, 1H)	10

Table 12. ^{13}C-nmr of 8-ethyl-5,7-dihydroxy-2-methylchroman-4-one (Acetone-d_6, 100.6 MHz).

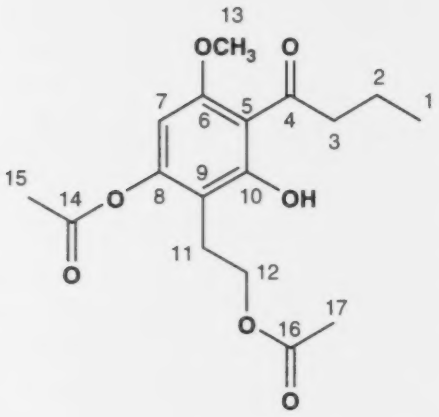
Signal δ	Assignment
13.4	2'
20.3	9
22.3	1'
45.9	3
73.5	2
90.9	6
104.2	4a
109.6	8
155.0	5
154.5	7
161.5	8a
191.7	4

II. B. 3. Derivatives

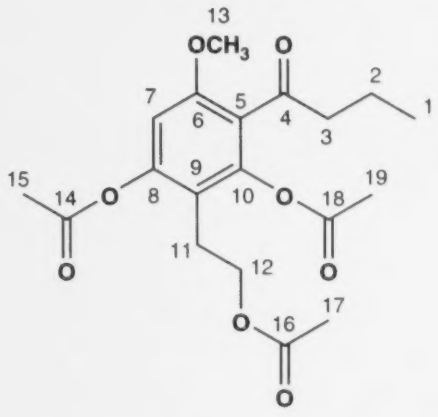
II. B. 3. 1. Acetylation of Phomalone

Treatment of phomalone with acetic anhydride-triethylamine in methylene chloride gave a mixture of two crystalline compounds. One of them gave a color reaction with ferric chloride and shows a strong molecular ion peak at m/z 338 in the hreims, for a molecular formula $C_{17}H_{22}O_7$. This indicates the addition of two acetyl groups to phomalone ($C_{13}H_{18}O_5$). The second compound, which did not give a color reaction with ferric chloride, shows a

molecular ion at m/z 380 with a molecular formula $C_{19}H_{24}O_8$. This suggests the incorporation of three acetyl groups into phomalone. These two derivatives are assumed to have the structures **35** and **30** respectively.



35



30

The ^{1}H-nmr spectral data for compounds **35** and **30** show the presence of the structural units of phomalone, with the addition of two singlets for **35** (δ 2.36 and δ 2.05), and three singlets for **30** (δ 2.40, δ 2.27 and δ 2.05). These signals arise from the hydrogens of the acetoxy groups. Analogous to phomalone, homodecoupling and INAPT experiments established the carbon skeleton, and allowed the assignment of structures **35** and **30**. The relative position of the methoxy group and the hydrogen in the aromatic ring was determined by a NOE experiment. The hydrogen signal for the methoxy group at δ 3.82 (in **30**) exhibits 3.6% enhancement upon irradiation of the aromatic proton at δ 6.13, while the aromatic hydrogen (in **30**) shows a 2.9% enhancement upon irradiation of the hydrogens of the methoxy group. The presence of a carbonyl *ortho* to a phenolic moiety for phomalone diacetate is confirmed by a singlet at δ 14.05 in the ^{1}H-nmr spectrum. Tables 13, 14, 15, and 16 summarize the final assignment of the nmr data of structures **35** and **30**.

Table 13. ^{1}H-nmr of phomalone diacetate ($CDCl_3$, 400 MHz).

Signal δ (m, area, J)	Assignment
0.99 (t, 3h, J=7.0 Hz)	1
2.05 (s, 3H)	15[a]
1.71 (tq, 2H, J=J'=7.0 Hz)	2
2.36 (s, 3H)	17[a]
2.86 (t, 2H J=7.0 Hz)	3
3.01 (t, 2H, J=7.0 Hz)	11
3.87 (s, 3H)	13
4.18 (t, 2H, J=7.0 Hz)	12
6.15 (s, 1H)	7
14.05 (s, 1H)	18

a: assignment may be interchanged

Table 14. ^{13}C-nmr for phomalone diacetate ($CDCl_3$, 100.6 MHz).

Signal δ	Assignment
13.9	1
17.8	2
20.9	17[a]
21.0	15[a]
22.7	11
46.6	3
55.7	13
62.8	12
90.9	7
96.3	5
103.4	9
109.0	8
111.1	10
155.0	6
166.9	14[b]
169.0	16[b]
207.2	4

a, b: assignments may be interchanged.

Table 15. ^{1}H-nmr of phomalone triacetate ($CDCl_3$, 400 MHz).

Signal δ (m, area, J)	Assignment
0.95 (t, 3H, J=7.0 Hz)	1
1.66 (tq, 2H, J=7.0 Hz)	2
2.05 (s, 3H)	15[a]
2.27 (s, 3H)	19[a]
2.40 (s, 3H)	17[a]
2.72 (t, 2H, J=7.0 Hz)	3
2.79 (t, 2H, J=7.0 Hz)	11
3.82 (s, 3H)	13
4.10 (t, 2H, J=7.0 Hz)	12
6.13 (s, 1H)	7

a: assignments may be interchanged.

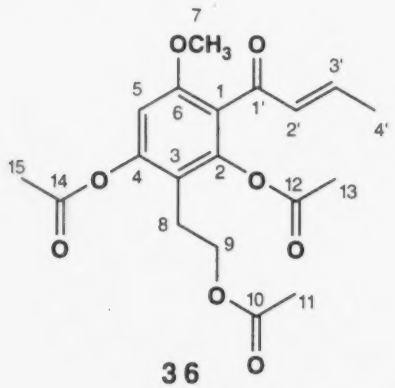
Table 16. ^{13}C-nmr for phomalone triacetate ($CDCl_3$, 100.6 MHz) .

Signal δ	Assignment
13.7	1
17.2	2
20.6	19[a]
20.9	15, 16[a]
24.2	11
45.9	3
56	13
62.7	12
104.2	7
116.0	5
122.6	9
147.5	6
151.4	8
156.3	10
166.9	14[b]
169.0	16[b]
170.9	18[b]
202.7	4

a, b: assignments may be interchanged.

II. B. 3. 2. Acetylation of a crude sample to afford (*E*)-2,4-diacetoxy-3-(2-acetoxyethyl)-6-methoxy-2'-en-butyrophenone

Acetylation of a crude sample obtained from the extraction of HP-20 resin gave a clear oil. The ir spectrum shows carbonyl absorptions: 1771 cm^{-1} (C=O, ester), 1738 cm^{-1} (C=O, ester), and 1660 cm^{-1}(C=O, ketone). This low frequency for the ketone absorption, and another absorption at 968 cm^{-1} for a carbon-carbon double bond, suggested an α,β –unsaturated ketone. The ^{1}H-nmr spectrum shows two methylene groups, one at δ 2.71 (consistent with benzylic hydrogens) and the other at δ 4.11 (methylene attached to an oxygen atom). A methoxy group is revealed by a donwfield singlet (δ 3.77). A singlet at δ 6.64 suggests an aromatic hydrogen, and two olefinic hydrogens at δ 6.34 and δ 6.75 confirm the α,β–unsaturated ketone. The remaining signal corresponds to a methyl group at δ 1.92. The low field position for this signal suggests that the methyl group is linked to the unsaturated system. A coupling constant for the olefinic protons (J=16.5 Hz) suggests a *trans*-configuration of the double bond. Homodecoupling experiments confirm these observations and allow the assignment of a *trans*-configuration of the double bond. The olefinic hydrogens at δ 6.75 and δ 6.34 become doublets (J= 16.5 Hz) upon irradiation of the methyl group at δ 1.92, and the doublet at δ 1.92 becomes a singlet upon irradiation of the hydrogen at δ 6.75. The ^{13}C-nmr spectrum shows a similar set of signals with those of phomalone triacetate. Signals at δ 132.5 and δ 146.9 are consistent with the α,β–unsaturated ketone. Structure **36** is assigned to this derivative. The nmr data are summarized in Tables 17 and 18.



36

Table 17. ^{1}H-nmr of (*E*)-2,4-diacetoxy-3-(2-acetoxyethyl)-6-methoxy-2'-en-butyrophenone ($CDCl_3$, 400 MHz).

Signal δ (m, area, J)	Assignment
1.92 (dd, 1H, J=7.0 Hz)	4'
2.03 (s, 3H)	13[a]
2.23 (s, 3H)	15[a]
2.38 (s, 3H)	11[a]
2.71 (t, 3H, J=7.5 Hz)	8
3.77 (s, 3H)	7
4.11 (t, 3H, J=7.5 Hz)	9
6.34 (dq, 1 H, J=16.5 Hz, J'=2.0 Hz)	2'
6.75 (dq, 1H, J=16.5 Hz, J'=7.0 Hz)	3'
6.64 (s, 1H)	5

a: assignments may be interchanged.

Table 18. ^{13}C-nmr of (*E*)-2,4-diacetoxy-3-(2-acetoxyethyl)-6-methoxy-2'-en-butyrophenone ($CDCl_3$, 400 MHz).

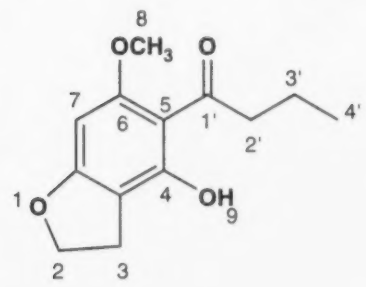
Signal δ	Assignment
17.2	4'
20.9	15[a]
21.0	11, 13[a] (overlapped)
23.9	8
56.0	7
62.6	9
104.3	5
115.9	1
120.9	3
132.5	3'
146.9	2'
147.6	6
151.3	4
168.9	10[b]
169.1	14[b]
170.9	12[b]
156.3	2
202.7	1'

a, b: assignments may be interchanged.

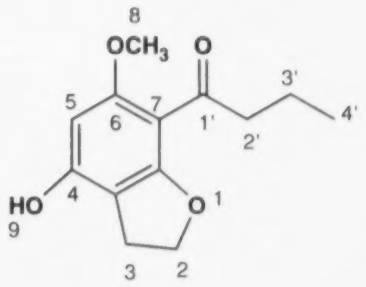
II. B. 3. 3. Cyclization reactions

Treatment of phomalone with *p*-toluenesulfonic acid or *p*-toluenesulfonyl chloride afforded a mixture of two white crystalline compounds. In the former reaction, *p*-toluenesulfonic acid monohydrate (1 eq.) was added to a solution of phomalone in benzene. After stirring at room temperature of 1.5 h, another 2 eq. of acid was added and the mixture was refluxed for 4.5 h with continuous removal of water (Dean-Stark). In the second reaction, *p*-toluenesulfonyl chloride (6 eq.) was added to a solution of phomalone in benzene. The mixture was refluxed for 4.5 h.

The two products each gave a color reaction with ferric chloride and show in the hreims molecular ion peaks at m/z 236, for a molecular formula $C_{13}H_{16}O_4$ in each case. The ir data shows a low frequency carbonyl absorption (1645 cm^{-1}) and indicates the presence of a hydroxyl group (3200 cm^{-1}). The ^{1}H-nmr spectrum for both compounds show similar signals except for the position of a singlet at low field. One compound shows a signal at δ 14.5, while the second one shows it at δ 9.5. The low field absorption suggests the presence of a phenolic moiety chelated with an *ortho* carbonyl group in one case, and an free phenolic (OH) moiety in the other. Moreover, the spectroscopic data for one of these compounds matched those of an authentic sample of the natural product 4-hydroxy-6-methoxy-5-(1'-oxobutyl)benzo[b]dihydrofuran (**31**). Therefore, one product has structure **31**. This is consistent with the signal at δ 14.5 in the ^{1}H-nmr spectrum.



31



32

The second compound is assigned structure **32**, with the free phenolic OH (δ 9.5). The nmr data are summarized in tables 7 and 8 for **31**, and 19 and 20 for **32**.

Table 19. ^{1}H-nmr of 4-hydroxy-6-methoxy-7-(1'-oxobutyl)benzo[b]dihydrofuran ($CDCl_3$, 400 MHz).

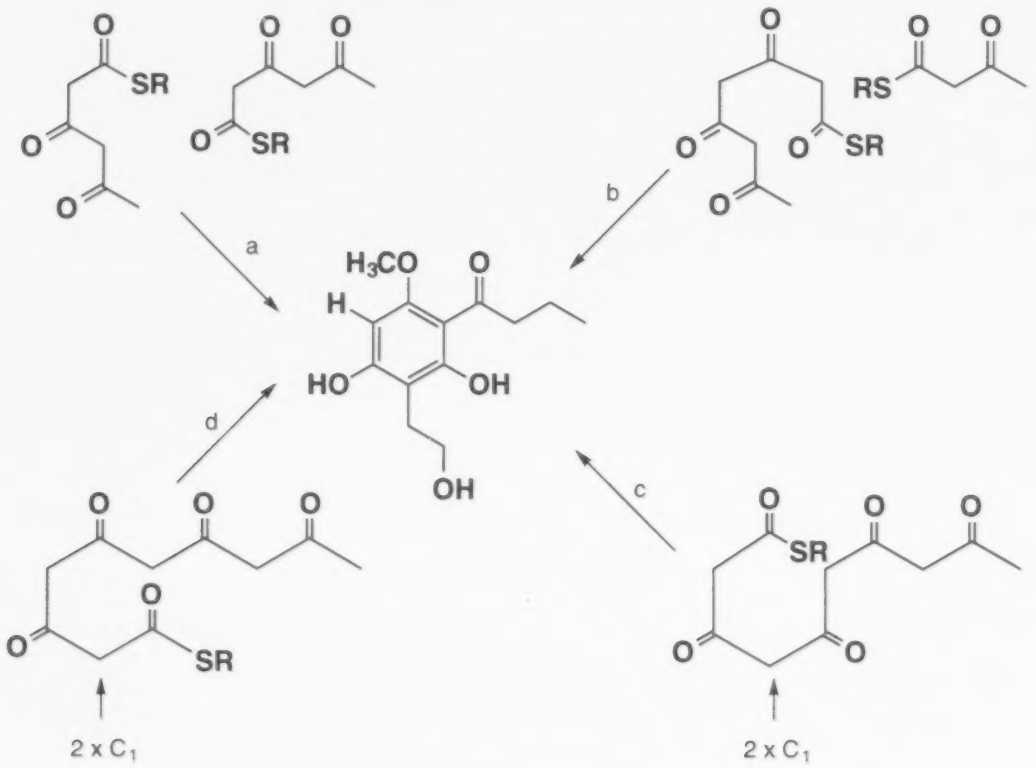
Signal δ (m, area, J)	Assignment
0.98 (t, 3H, J=7.0 Hz)	4'
1.60 (qt, 2H, J=J'=7.0 Hz)	3'
2.80 (t, 2H, J=7.0 Hz)	2'
3.20 (t, 2H, J=7.0 Hz)	3
3.80 (s, 3H)	8
4.50 (t, 2H, J=7.0 Hz)	2
5.90 (s, 1H)	5
9.50 (s, 1H)	9

Table 20. ^{13}C-nmr of 4-hydroxy-6-methoxy-7-(1'-oxobutylbenzo[b]dihydrofuran ($CDCl_3$, 100.6 MHz).

Signal δ	Assignment
14.2	4'
18.9	3'
26.4	3
46.6	2'
55.9	8
62.9	2
92.4	5
132.9	7
142.6	3a
162.7	6
164.3	7a
165.9	4
206.4	1'

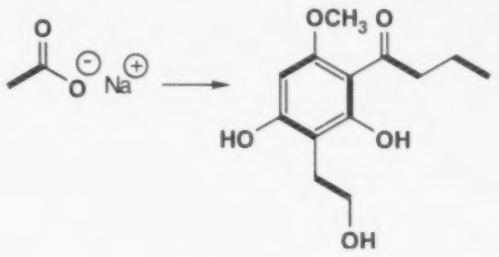
II. C. BIOSYNTHESIS

Structural analysis suggests that phomalone is a polyketide derivative. However, the carbon skeleton cannot be formed by simple folding of a single polyketide intermediate. Some possible biosynthetic pathways are shown in Scheme 5. Two possibilities arise from the condensation of two preformed polyketide chains: two triketide precursors (a) and one tetraketide and one diketide (b). Another possibility is that the side chain is formed by two successive C-methylations (or incorporation of two carbon unit at either position 2 or 4) of a pentakide precursor which can fold in two possible ways (c and d).



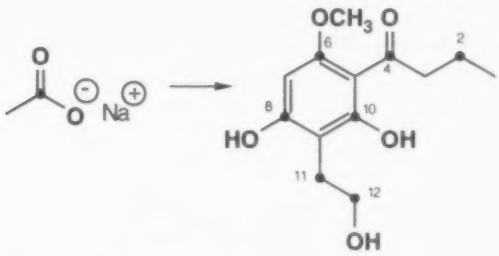
Scheme 5

The incorporation of [1,2-$^{13}C_2$]-sodium acetate showed that the carbon skeleton is formed from six intact acetate units (Scheme 6).



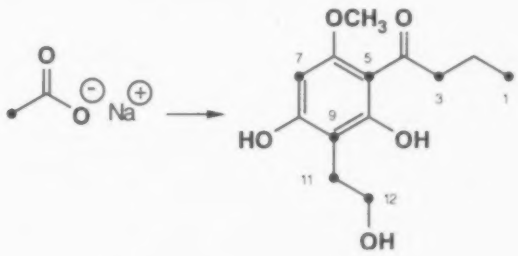
Scheme 6

Incorporation of [1-^{13}C]-sodium acetate (Scheme 7) gave labeling not only at the normally oxygenated carbons of the polyketide chain, but also at C12. C11 showed more enrichment than C12.



Scheme 7

Incorporation of [2-^{13}C]-sodium acetate (Scheme 8) gave similar results. In this case, C12 showed higher enrichment than C11.

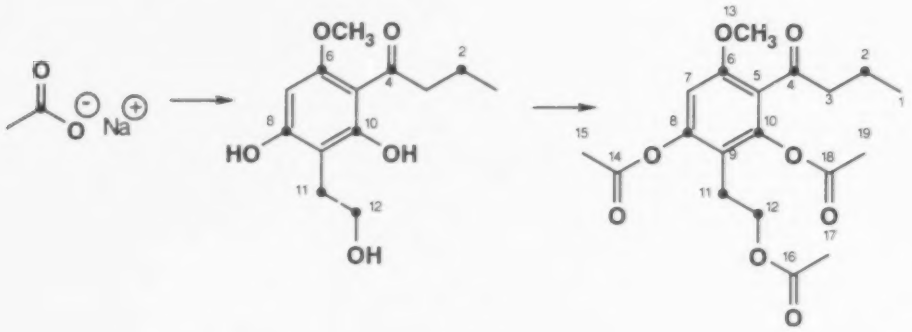


Scheme 8

The incorporation of both monolabelled [^{13}C]-sodium acetates were evaluated on the triacetate derivative. Normalization of the ^{13}C-nmr enrichment was established using the signal for the methyl from the acetoxy groups as the measure of natural abundance. The enrichment was calculated according to the following formula:[70]

$$\text{Percentage Enrichment} = \left(\frac{\text{Observed } ^{13}\text{C Abundance}}{\text{Natural } ^{13}\text{C Abundance}} \right) - 1.1$$

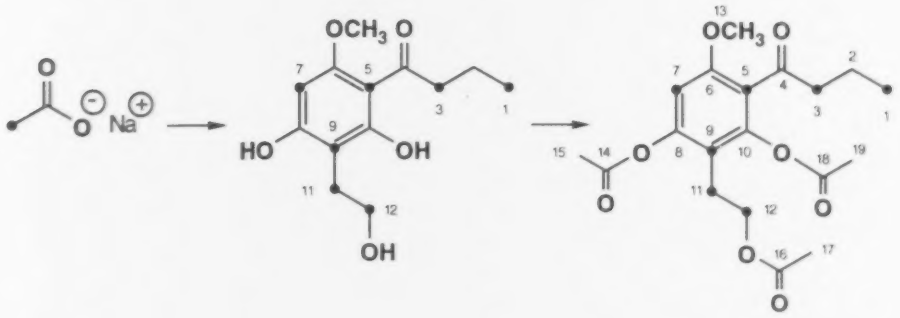
Table 21 and Scheme 9 show the incorporation of [1-^{13}C]-sodium acetate. Similarly, Table 22 and Scheme 10 describe the results for the corresponding [2-^{13}C]-sodium acetate. Scheme 11 describes [1,2-$^{13}C_2$]-sodium acetate incorporation.



Scheme 9

Table 21. ^{13}C-nmr of phomalone triacetate after feeding with [1-^{13}C]-sodium acetate ($CDCl_3$, 100.6 MHz).

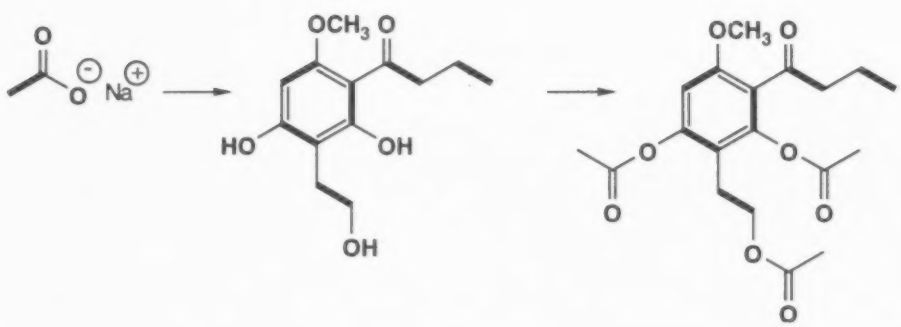
Carbon	δ	Carbon % enrichment
2	17.18	3.2
4	202.62	3.5
6	147.46	1.7
8	151.34	2.7
10	156.26	2.7
11	24.10	4.9
12	62.65	1.1



Scheme 10

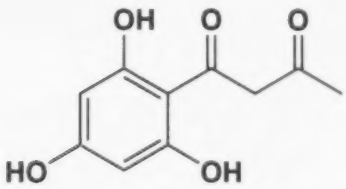
Table 22. ^{13}C-nmr of phomalone triacetate after feeding with sodium [2-^{13}C]-sodium acetate ($CDCl_3$, 100.6 MHz).

Carbon	δ	Carbon % enrichment
1	13.70	7.9
3	45.82	6.7
5	115.92	8.8
7	104.18	5.8
9	122.56	4.2
11	24.26	1.3
12	62.66	4.6

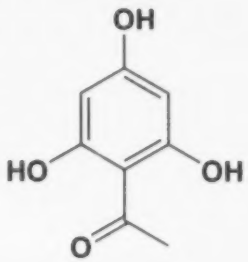


Scheme 14

These results eliminated pathways c and d of Scheme 5 because they require two successive C-methylations of a pentaketide precursor. The carbon skeleton is derived from six intact acetate units. The incorporation of [1,2-$^{13}C_2$]-sodium acetate suggested that intermediates containing a symmetrically substituted phoroglucinol ring like **37** or **38** were unlikely. Biosynthetic studies have confirmed that such symmetrical intermediates afford randomization of labeling in the phloroglucinol ring or, similarly, in resorcinol.[71]

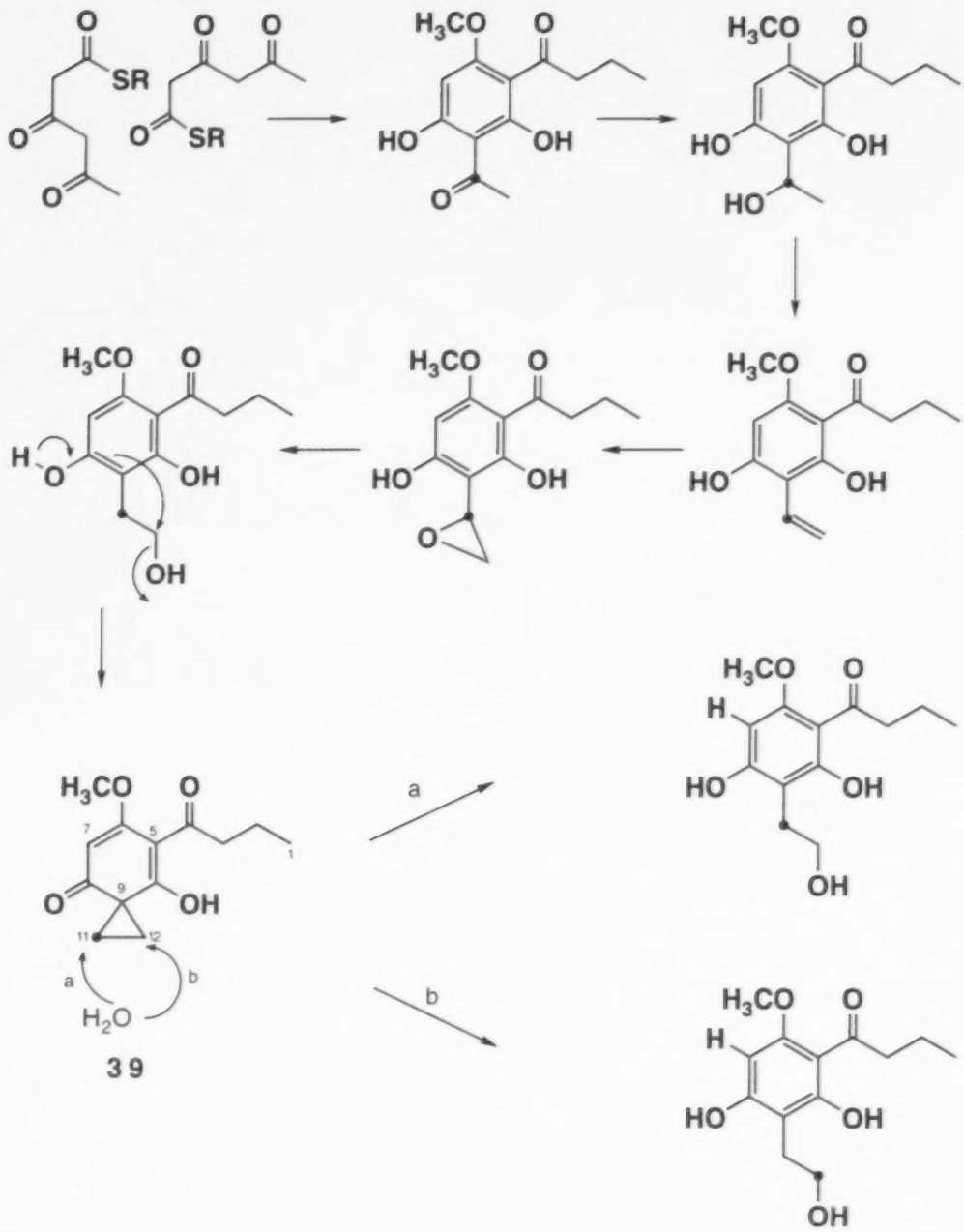


37



38

The randomization of labeling on the side chain was unexpected. However, it is likely that these carbons become equivalent during the biosynthesis. Formation of the cyclopropyl intermediate **39** by participation of the aromatic ring is consistent with this observation.[72] Scheme 12 shows the proposed biosynthesis of phomalone.



39

Scheme 12

II. D. CONFIRMATORY BIOASSAYS

The multiwell method was found to be the best bioassay method for the crude extracts and standard solutions of phomalone. An aliquot of a standard solution of phomalone was applied directly to the well of the multiwell plate (six well, flat bottom) using a micropipette. Three replicates for each sample and the solvent (control) were evaluated in each multiwell plate. Radial growth was measured 6, 14 and 21 days after inoculation by dividing by two the mean o two diameter readings of each plate taken at right angles each other. This procedure was repeated for seven different concentrations: $4 \cdot 10^{-3}$, $2 \cdot 10^{-3}$, $1 \cdot 10^{-3}$, $4 \cdot 10^{-4}$, $2 \cdot 10^{-4}$, $1 \cdot 10^{-4}$, and $4 \cdot 10^{-5}$ mol·L^{-1}. The bioassay was evaluated by the amount of growth of *P. tremulae*, relative to the control, in each well. Tables 23, 24 and 24 summarize the bioassays.

Table 23. Bioassay of phomalone using 1 mL-doses.

Concentration mol·L^{-1}	Inhibition %
$4 \cdot 10^{-3}$	100
$2 \cdot 10^{-3}$	100
$1 \cdot 10^{-3}$	100
$4 \cdot 10^{-4}$	100
$2 \cdot 10^{-4}$	53
$1 \cdot 10^{-4}$	33
$4 \cdot 10^{-5}$	0[a]

a: No inhibition compared to the control

Table 24. Bioassay of phomalone using 0.5 mL-doses.

Concentration mol·L^{-1}	Inhibition %
$4 \cdot 10^{-3}$	100
$2 \cdot 10^{-3}$	100
$1 \cdot 10^{-3}$	100
$4 \cdot 10^{-4}$	48
$2 \cdot 10^{-4}$	30
$1 \cdot 10^{-4}$	0[a]
$4 \cdot 10^{-5}$	0[a]

a: no inhibition compared to the control.

Table 25. Bioassay of phomalone using 0.25 mL-doses.

Concentration $mol \cdot L^{-1}$	Inhibition %
$4 \cdot 10^{-3}$	100
$2 \cdot 10^{-3}$	100
$1 \cdot 10^{-3}$	48
$4 \cdot 10^{-4}$	30
$2 \cdot 10^{-4}$	0[a]
$1 \cdot 10^{-4}$	0[a]
$4 \cdot 10^{-5}$	0[a]

a: No inhibition compared to the control.

From the results above, it was concluded that phomalone inhibits the growth of *P. tremulae* on MEA. Under these conditions, concentrations higher than 0.1 mg (as the total amount in each well) inhibited *P. tremulae* completely, while concentrations lower than 0.01 mg did not show any activity. Concentrations between these two values gave partial inhibition. It may be concluded that phomalone is reponsible for the antagonism shown between the growing cultures of *P. etheridgei* and *P. tremulae*. However, the quantitative bioassays indicate that this is not a spectacular activity. Further testing of the antifungal activity of phomalone will be carried out by CIBA-GEIGY Agricultural Division (Switzerland).

III. EXPERIMENTAL

III. A. GENERAL

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Infrared spectra (ir) were obtained using the following spectrophotometers: Nicolet 7199 FTIR, Nicolet MX-1 FTIR, and Nicolet 750 FTIR. Electron impact mass spectra (eims) were obtained using a Kratos AEI MS50 high resolution mass spectrometer and a Kratos AEI MS12 low resolution mass spectrometer. Chemical ionization mass spectra (cims) were recorded on an Kratos AEI MS-12 mass spectrometer with ammonia as the reagent gas. Ultraviolet (uv) spectra were recorded on a Hewlett Packard 8450A diode array spectrophotometer. Hydrogen nuclear magnetic resonance spectra (^{1}H-nmr) were obtained using the following spectrometers: Bruker WH-200 (200 MHz), Bruker AM-400 (400 MHz), and Varian Unity-500 (500 MHz). Coupling constants are reported within ±0.5 Hz. Carbon-13 nuclear magnetic resonance spectra (^{13}C-nmr) were obtained on a Bruker AM-300 (75 MHz) and Bruker AM-400 (100.6 MHz). Carbon-13 multiplicities were determined using spin echo J-modulated experiments (APT or Attached Proton Test).[73,74] Methyl and methine groups are shown as signals possessing opposite phase (o) with respect to the deuteriochloroform signal, whereas methylene, quaternary and carbonyl carbons appear in phase (p). Nuclear Overhauser Enhancement (NOE)[59] experiments were determined in the difference mode in which a control (unsaturated) spectrum was computer-subtracted from the irradiated spectrum after Fourier Transformation. Positive enhancements appear as signals possessing opposite phase with respect to the irradiated signal. Samples for NOE measurements were deoxygenated with argon gas for 10-20 minutes prior to use. Homonuclear decoupling experiments were performed by using the Bruker DISNMR software package. Insensitive Nuclei Assigned by Polarization Transfer (INAPT)[61-63] were performed using the Varian Unity-500. Carbon-13 nuclear magnetic resonance spectra (^{13}C nmr) of labeled material: [1-^{13}C]-phomalone, [2-^{13}C]-phomalone, [1,2-$^{13}C_2$]-phomalone and the corresponding triacetates were obtained on a Bruker AM-400 (100.6 MHz) spectrometer. Carbon-13 experiments on labeled material were performed using the Bruker DISNR software package. A

relaxation delay of four seconds gave the best relative intensity for the signal which is produced by the same kind of carbon bearing equal number of hydrogens. High Performance Medium Pressure Liquid Chromatography (HPMPLC) was carried out on ACE Glass Inc. Michel-Miller equipment by using a solvent pump from Fluid Metering Inc. model RP-SY. Compounds were detected by using the ISCO V4 wavelength absorbance detector, and fractions were collected with the ISCO fraction collector model 820. High Pressure Liquid Chromatography (HPLC) analyses were performed on a Waters 600E System Controller equipped with a 490E Programmable multiwavelength U.V. detector, and M730 Data Module. Liquid cultures were grown under shaking conditions on an orbital shaker, New Brunswick Scientific model G-53 at 150 rpm. Petri dishes cultures were blended in a Waring blender. The cultures were sterilized in an Autoclave AMS-50, Lab. Isothermal. The liquid cultures were freeze-dryed using the Freeze-dryer Labconco 4.5 L. *P. etheridgei* spores were counted with a hemacytometer (graduated surface normally used to count blood cells) under a Fisher Scientific microscope equipped with a reichert wien Nr. 223537.

III. B. MATERIALS

Flash chromatography was performed using silica gel of 230-400 mesh. HPMPLC was carried out on columns filled with silica gel Merck Lichroprep Si60, 20-40 µm (normal phase); Merck Lichroprep RP-18, 20-40 µm (reversed-phase). Thin layer chromatography was performed on Merck aluminum-backed plates precoated with silica gel 60 GF_{254}, 0.2 mm thickness. The chromatograms were examined under UV-light at 254 nm, and visualization was completed by dipping into a 2% vanillin acid solution (2 g of vanillin, 5 mL concentrated H_2SO_4, diluted to 250 mL with 95% ethanol). Preparative thin layer chromatography was performed on Merck glass plates with Silica gel 60 F_{254}, 0.25 mm thickness. HPLC-samples were prepared using Waters Sep-pak™ cartridges, C-18 and NH_2 solid phase extraction. HPLC analyses were performed in the following columns: steel columns, µ Bondapack™ C-18, 3.9·300 mm, 125Å, 10 µm; µ Bondapak™ C-18 7.8·300 mm,125Å, 10 µm; Radial-Pak™ cartridges, µ Bondapak™ NH_2, 8·10 mm, 10 µm, RCM™ 8x10 cartridge holder. Liquid media were prepared

using the following reagents: Bacto™ malt extract broth DIFCO laboratories; Bactopectone™ DIFCO laboratories; Agar (fine powder) DIFCO laboratories; Phytone™ yeast extract agar, BBL Microbiology Systems Becton Dickinson and Codkeysville; Phytone™ pectone (papaic digest of soybean meal), BBL Microbiology Systems Becton Dickinson and Codkeysville. Bioassays were carried out on multiwell™ tissue culture plate, six (Falcon™ 3047) and twenty four well flat bottom (Falcon™ 3046) with low evaporation lid (tissue culture treated polystyrene, sterilized by γ irritation, Becton-Dickinson and Company). A micropipette (Gilson 100 μL) was used to dispense samples for the bioassay. Feeding experiments were performed with [1-^{13}C]-sodium acetate, [2-^{13}C]-sodium acetate and [1,2-$^{13}C_2$]-sodium acetate which are provided by ICON Isotopes, ICON Services. HP-20 ion resin exchange was obtained from Mitsubishi, Porland, Ore. Solvents for the preparation of derivatives were purified as follows: methylene chloride by distillation over calcium hydride at atmospheric pressure; triethylamine by distillation first from potassium hydroxide and then from calcium hydride; acetic anhydride by distillation from calcium hydride; benzene was treated with concentrated sulfuric acid, washed in turn with 10% sodium bicarbonate, water, and then dried over calcium chloride, filtered and distilled from calcium hydride. Skellysolve B refers to Skelly oil company light petroleum (boiling point 62-70 °C). All solvents were distilled at atmospheric pressure prior to their use.

III. C. FUNGAL CULTURES

III. C. 1. Organisms

The two fungal isolates were obtained from the Northern Forest Research Center. They are deposited in the fungal culture collection at Northern Forestry Center, Forestry Canada, Edmonton (NOF) and the University of Alberta Microfungus Collection (UAMH).

Phoma etheridgei (NOF 1610, UAMH 7003) was isolated from the surface of a black gall of trembling aspen. *P. etheridgei* was maintained on malt extract agar medium in Petri dishes, and as a freeze dried material, until used.

Phellinus temulae (NOF 1464, UAMH 7005) was isolated from basidiomes associated with trembling aspen. *P. tremulae* was maintained in malt extract agar and carrot medium in Petri dishes until used.

III. C. 2. Solid media

P. etheridgei was inoculated on four different media: a) Malt extract agar (MEA), b) Phytone yeast extract agar (PYEA), c) Potato dextrose agar (PDA), and d) Potato dextrose yeast agar (PDYA). Table 26 describes these media in more detail.

Table 26. Petri dishes media

Medium	Components	Quantity
MEA	Malt extract broth	2 g
	Agar	2 g
	distilled water	1 L
PYEA[a]	Phytone ™yeast extract agar	72 g
	distilled water	1 L
PDA[b]	potato dextrose agar	24 g
	distilled water	1 L
PDYA	potato dextrose agar	24 g
	yeast extract	2 g
	distilled water	1 L

[a]This medium is commercially available from Baltimore Laboratories (BBL).

[b]This medium is commercially available from Difco.

III. C. 3. Liquid media

P. etheridgei was inoculated in five different liquid media: a) Malt medium (MM), b) Phytone yeast extract medium (PYEM), c) Potato dextrose yeast broth (PDYB), d) Potato dextrose broth (PDB), and e) Czapek's medium (CzM). These media are described in table 27.

Table 27. Liquid media

Medium	Component	Quantity
MM	glucose	20 g
	malt extract broth	20 g
	bacto-pectone	1 g
	water	1 L
PYEM[a]	phytone peptone™ (papaic digest of soybean meal)	10 g
	yeast extract	5 g
	glucose	20 g
	water	1 L
PDBY	potato dextrose broth	20 g
	yeast extract	2 g
	water	1 L
PDB[b]	potato dextrose broth	20 g
	water	1 L
CzM	glucose	20 g
	$NaNO_3$	3g
	K_2HPO_4	0.5 g
	KCl	0.5 g
	$FeSO_4$	0.01 g
	water	1 L

[a]This medium is commercially available from Baltimore Laboratories (BBL).

[b]This medium is commercially available from Difco.

III.C. 4. Inocula

Inocula for the liquid media were prepared by the following procedures: a) Early growth phase. After seven days, the MEA culture was blended with 200 mL of sterile water, and a 10 mL aliquot of the aqueous suspension of mycelium was used to inoculate the liquid medium. This procedure was repeated with PYEA, PDA, PDYA Petri dishes.

b) Old growth phase. After fifteen days, the MEA culture was blended with 200 mL of sterile water, and a 10 mL aliquot of the aqueous suspension of mycelium was used to inoculate the liquid media. This procedure was repeated with PYEA, PDA, PDYA Petri dishes.

c) Spore suspension. After thirty days, the spores were counted using a hemacytometer. The MEA culture was blended with 200 mL of sterile water, and the resulting aqueous suspension was adjusted to 10000-20000 spores·mL^{-1}. A 3 mL aliquot of this spore suspension was used as inoculum for one liter of liquid media. This procedure was repeated with PYEA media.

III. C. 5. Inoculation of liquid cultures

Liquid cultures were prepared in 10 L batches by using one of the three inocula. An aqueous suspension of the inoculum was used to inoculate 10·1L sterile MM-liquid medium in 2 L-Erlenmeyer flasks. After inoculation, the liquid cultures were kept at room temperature for twelve days. *Phoma etheridgei* was grown in still and shake culture. This procedure was repeated with PYEM, PDBY, PDB, and CzM liquid media.

III. C. 6. Liquid cultures with HP-20 resin

Liquid cultures (10 L)were prepared using spore suspension as inoculum. A 3 mL aliquot of the aqueous suspension containing

10000-20000 spores·mL^{-1} was used to inoculate 10·1L sterile MM-liquid medium in 2 L Erlenmeyer flasks. HP-20 resin (20 g·L^{-1}) was added to MM-liquid medium. After inoculation, the liquid cultures were kept at room temperature for twelve days under shaking conditions. This procedure was repeated with PYEM-liquid medium.

III. C. 7. Feeding experiments

Liquid cultures (2 L) were prepared using the spore suspension as inoculum. A 3 mL aliquot of the aqueous suspension containing 10000-20000 spores·mL^{-1} was used to inoculate 2·1L sterile MM-liquid medium in 4 L-Erlenmeyer flasks. HP-20 ion exchange resin (20 g·L^{-1}) was added to MM-liquid medium. The liquid cultures were kept at room temperature under shaking conditions. After three days, a solution of [1-^{13}C]-sodium acetate (15 mL, 0.2 mol·L^{-1}) was added under sterile conditions. Two further additions were made on the fifth and seventh day. *P. etheridgei* was grown under shaking conditions for a total of thirteen days. The same procedure was used with [2-^{13}C]-sodium acetate and [1,2-$^{13}C_2$]-sodium acetate.

III. D. ISOLATION PROCEDURES

III. D. 1. Crude extracts from liquid cultures

This section describes the procedures used to obtain the crude extracts that were evaluated in the bioassay against the growth of *P. tremulae* in MEA-Petri dishes. These procedures were repeated for MM, PYEM, PDBY, PDB, and CzM liquid media.

The culture broth of *P. etheridgei* was separated from the mycelium by filtration through cheese cloth. The mycelium was air-dried, pulverized, and subjected to consecutive Soxhlet extractions with methylene chloride, ethyl acetate and methanol. The organic extract from each solvent was dried over

anhydrous sodium sulfate and concentrated under reduced pressure to give a crude mycelium extract.

The broth from each batch was combined and extracted according to the following procedures:

a) The culture broth (10 L) was concentrated under reduced pressure (vacuum pump, temperature lower than 40 °C) to about 1 L, and extracted with ethyl acetate (3·1 L) by stirring for twenty four hours at room temperature. The ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure.

b) The culture broth (10 L) was freeze-dried to give a residue which was extracted with methanol (3·500 mL). After vacuum filtration, the extract was concentrated under reduced pressure.

c) The culture broth (1 L) was acidified to pH 3 with hydrochloric acid (1 $mol \cdot L^{-1}$), and extracted with ethyl acetate (3·250 mL) by stirring for six hours at room temperature. The ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure.

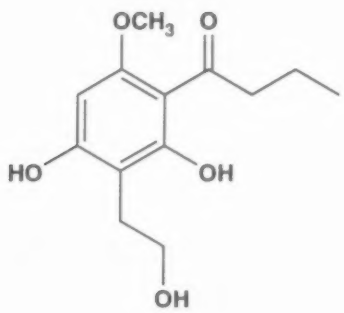
d) The culture broth (1 L) was adjusted to pH 9 with sodium hydroxide (1$mol \cdot L^{-1}$), and extracted with ethyl acetate (3·250 mL) by stirring for six hours at room temperature. The ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure.

III. D. 2. Crude extracts from liquid cultures treated with HP-20 resin

In contrast to the previous liquid cultures, the extraction procedure was performed on the resin rather than on the broth. The floating HP-20 ion exchange resin was decanted from the culture. The remaining resin which adhered to the mycelium was separated by vacuum filtration through Whatman paper No.1. The resin and the mixture resin-mycelium were separately

transferred to a glass-filter (porosity D, ASTM 10-20 μm) and extracted with ethyl acetate (4·400 mL). The ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give a viscous brown oil.

III. E. ISOLATION OF PHOMALONE [2,4-dihydroxy-3-(2-hydroxyethyl)-6-methoxybutyrophenone (**23**)]



III. E. 1. PYE-liquid culture

The broth of a PYEM-liquid culture (7 L, 12 days shaking) was concentrated under reduced pressure (vacuum pump, temperature lower than 40 °C) to about 1 L, and extracted with ethyl acetate (3·1 L) by stirring for twenty four hours at room temperature. The ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give the broth extract (0.97 g) as a viscous brown oil. Flash chromatography of 432 mg of the broth extract (10% ethyl acetate in methylene chloride) gave impure phomalone (57 mg). Flash chromatography of this sample (10% ethyl acetate in methylene chloride) followed by HPMPLC (RP-18, 30% water in methanol) afforded phomalone, 2,4-dihydroxy-3-(2-hydroxyethyl)-6-methoxybutyrophe–none (35 mg): mp 126-127 °C; R_f=0.25 (10% ethyl acetate in methylene chloride); ^{1}H nmr (400 MHz, acetone-d_6) δ 14. 50 (s, 1H, Ar-O**H**), 6.01 (s, 1H, Ar-**H**), 3.85 (s, 3H, -OC**H**$_3$), 3.70 (t, 2H, J=7.0 Hz, -C**H**$_2$-CH$_2$-OH), 2.80 (t, 2H, J=7.0 Hz, O=C-C**H**$_2$-), 2.74 (t, 2H, J=7.0 Hz, Ar-C**H**$_2$-), 1.66 (tq, 2H, J=J'=7.0 Hz, -CH$_2$-C**H**$_2$-CH$_3$), and 0.95 (t, 3H, J=7.0 Hz, -C**H**$_3$); ir ($CHCl_3$, cast) 3200 (OH), 1618 (ketone C=O), and 1448, 1427 cm^{-1} (Ar-H); uv (EtOH)

205, 211, and 293 nm; hreims, m/z 254.1154 M^+ (calcd. for $C_{13}H_{18}O_5$ 254.1158), 223.2343 (100), 211.2345 (93), 193.1111 (63); ^{13}C nmr (100.6 MHz, acetone-d_6) δ 206.4 (p), 165.9 (p), 164.3 (p), 162.7 (p), 106.9 (p), 105.6 (p), 92.1 (o), 62.9 (p), 55.9 (o), 46.6 (p), 26.3 (p), 16.9 (p), and 14.2 (o).

III. E. 2. MM-liquid medium without resin

The broth of a MM-liquid culture (10 L, 12 days shaking) was concentrated under reduced pressure (vacuum pump, temperature lower than 40 °C) to about 1 L, and extracted with ethyl acetate (3·1 L) by stirring for 24 h at room temperature. The ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give the broth extract (0.78 g) as a viscous brown oil. Flash chromatography of 475 mg of the broth extract (10% ethyl acetate in methylene chloride) followed by HPMPLC (NP-Si 60, 10% ethyl acetate in methylene chloride), and HPMPLC (RP-18, 30% water in methanol) gave phomalone (8 mg). Properties are described in section III. E. 1.

III. E. 3. MM-liquid medium with resin

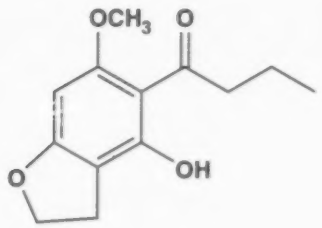
The floating HP-20 ion exchange resin was decanted from the broth of MM-liquid culture (2 L, 12 days). The remaining resin which adhered to the mycelium was separated by vacuum filtration through Whatman paper No.1. The resin and the mixture resin-mycelium were separately transferred to a glass-filter (porosity D, ASTM 10-20 μm) and extracted with ethyl acetate (4·400 mL). The ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduce pressure to give a resin crude extract (0.457 g). Flash chromatography of the resin extract (10% ethyl acetate in methylene chloride) gave an impure sample (0.10 g). HPMPLC (NP-Si60, 10% ethyl acetate on methylene chloride) followed by HPMPLC (RP-18, 30% water in methanol) gave phomalone (75 mg). Properties are described in section II. E. 1.

III. F. PHOMALONE RELATED COMPOUNDS

III. F. 1. Crude organic extract

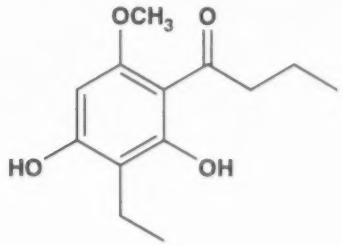
The broth of MM-liquid culture (2 L, 14 days) was extracted with ethyl acetate (3·1 L) by stirring for 24 h at room temperature. The ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give the broth extract (0.39 g) as a viscous-brown oil. Chromatographic separations of this crude organic extract afforded the following compounds.

III. F. 2. 4-Hydroxy-6-methoxy-5-(1'-oxobutyl)benzo[b]dihydrofuran (**31**)



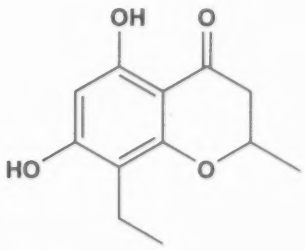
Flash chromatography of the organic extract (10% ethyl acetate in methylene chloride) gave 4-hydroxy-6-methoxy-5-(1'-oxobutyl)benzo[b]dihydrofuran as a white solid (4 mg): R_f=0.30 (2% ethyl acetate in methylene chloride); ^{1}H-nmr (400 MHz, $CDCl_3$) δ 14.50 (s, 1H, Ar-O**H**), 5.90 (s, 1H, Ar-**H**), 4.5 (t, 2H, J=7.0 Hz, -O-C**H**$_2$-C**H**$_2$-), 3.8 (s, 3H, -OC**H**$_3$), 3.2 (t, 2H, J=7.0 Hz, -CH$_2$-C**H**$_2$-Ar), 2.80 (t, 2H, J=7.0 Hz, O=C-C**H**$_2$-), 1.60 (tq, 2H, J=J'= 7.0 Hz, -CH$_2$-C**H**$_2$-CH$_3$), and 0.98 (t, 3H, J=7.0 Hz, -CH$_2$-C**H**$_3$); ir ($CHCl_3$, cast) 3250 (OH), 1650 (ketone C=O), 1450, 1497 cm^{-1} (Ar-H); hreims, m/z 236.1055 M$^+$ (calcd. for $C_{13}H_{16}O_4$ 236.1062), 193.0501 (100), 149.0241 (30); ^{13}C-nmr (100.6 MHz, acetone-d_6) δ 206.4 (p), 165.9 (p), 164.3 (p), 162.7 (p), 142.6 (p), 132.9 (p), 92.4 (o), 62.9 (p), 55.9 (o), 46.6 (p), 26.4 (p), 18.9 (p), and 14.2 (o).

III. F. 3. 3-Ethyl-2,4-dihydroxy-6-methoxybutyrophenone (**33**)



Flash chromatography of the organic extract (10% ethyl acetate in methylene chloride) following by prep-TLC (30% ethyl acetate in Skelly B) gave 3-ethyl-2,4-dihydroxy-6-methoxybutyrophenone as a white solid (R_f=0.47, 6 mg): ^{1}H-nmr (400 MHz, $CDCl_3$) δ 14.35 (s, 1H, Ar-O**H**), 5.90 (s, 1H, Ar-**H**), 3.52 (s, 3H, -OC**H**$_3$), 2.98 (t, 2H, J=7.0 Hz, O=C-C**H**$_2$), 2.59 (q, 2H, J=7.0 Hz, -C**H**$_2$-CH$_3$), 1.70 (tq, 2H, J=J'=7.0 Hz, -CH$_2$-C**H**$_2$-CH$_3$), 1.12 (t, 3H, J=7.0 Hz, -CH$_2$-C**H**$_3$), and 0.98 (t, 3H, J=7.0 Hz, -CH$_2$-C**H**$_3$); ir ($CHCl_3$, cast) 3200 (OH), 1618 (ketone C=O), and 1449, 1430 cm^{-1}(Ar-H); hreims, m/z 238.1207 M$^+$ (calcd. for $C_{13}H_{18}O_4$ 238.1209), 195.0661 (100); ^{13}C-nmr (100.6 MHz, $CDCl_3$) δ 202.3 (p), 164.9 (p), 161.5 (p), 159.6 (p), 109.5 (p), 105.7 (p), 90.1 (o), 55.4 (p), 46.3 (p), 22.3 (p), 18.2 (p), 14.0 (o), 13.4 (o).

III. F. 4. 8-Ethyl-5,7-dihydroxy-2-methylchroman-4-one (**34**)

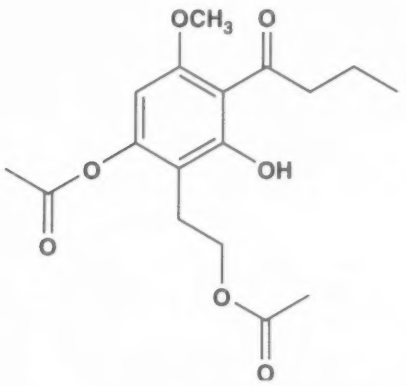


Flash chromatography of the organic extract (20% ethyl acetate in methylene chloride) following by HPMPLC (NP, Si60, 2% ethyl acetate in methylene chloride), and prep. TLC (2% ethyl acetate in methylene chloride) gave 8-ethyl-5,7-dihydroxy-2-methylchroman-4-one as a white solid (R_f 0.30, 1.3 mg):

^{1}H-nmr (400 MHz, acetone-d_6) δ 12.50 (s,1H, Ar-**OH**), 9.50 (s, 1H, Ar-**OH**), 6.10 (s, 1H, Ar-**H**), 4.50 (m, 1H, -**CH**), 2.60 (m, 4H, -CH_2-), 1.4 (d, 3H, -CH-CH_3, J=7.0 Hz), and 1.09 (t, 3H, -CH_2-CH_3, J=7.0 Hz); ir ($CHCl_3$, cast) 3133 (-OH), 1631 (ketone C=O), and 1467, 1451 cm^{-1} (Ar-H); hreims, m/z 222.08944 M^+ (calcd. for $C_{12}H_{14}O_4$ 222.08920), 207.06679 (100), 165.01921 (50); ^{13}C-nmr (acetone-d_6, 100.6 MHz) δ 191.7 (p), 161.5 (p), 154.5 (p), 155 (p), 109.6 (p), 104.2 (p), 90.9 (o), 73.5 (o), 45.9 (o), 22.3 (p), 20.3 (o), 13.4 (o).

III. G. Derivatives

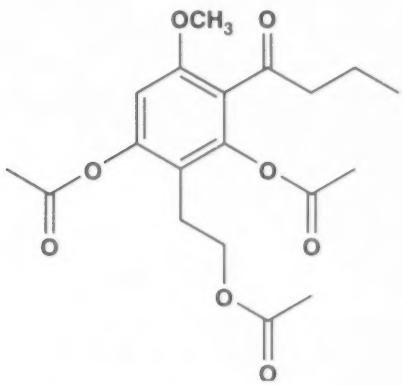
III. G. 1. Phomalone diacetate [4-acetoxy-3-(2-acetoxyethyl)-2-hydroxy-6-methoxybutyrophenone (**35**)]



Triethylamine (0.2 mL, 0.15 mmol, 3.3 eq.), and acetic anhydride (0.2 mL, 0.2 mmol, 5 eq.) were added to a solution of phomalone (11 mg, 0.04 mmol) in methylene chloride (2 mL). After stirring the mixture for 2 h at room temperature, the solution was concentrated under reduced pressure and the residue was dissolved in chloroform (3 mL). Prep-TLC (10% ethyl acetate in methylene chloride) gave phomalone diacetate [4-acetoxy-3-(2-acetoxyethyl)-2-hydroxy-6-methoxybutyrophenone] (R_f=0.60, 11 mg, 75%) and phomalone triacetate [2,4-diacetoxy-3-(2-acetoxyethyl)-6-methoxybutyrophenone] (mp 82-83 °C R_f=0.35, 3.5 mg, 20%). Phomalone diacetate: ^{1}H-nmr (400 MHz, $CDCl_3$) δ 14.05 (s,1H, Ar-**OH**), 6.15 (s, 1H, Ar-**H**), 4.18 (t, 2H, J=7.0 Hz, -CH_2-O-), 3.87 (s, 3H, -OCH_3), 3.01 (t, 2H, J=7.0 Hz, Ar-CH_2-), 2.86

(t, 2H, J=7.0 Hz, O=C-C**H**$_2$), 2.36 (S, 3H, O=C-CH$_3$), 2.05 (S, 3H, O=C-CH$_3$), 1.71 (tq, 2H, J=J'=7.0 Hz, -CH$_2$-C**H**$_2$-CH$_3$), , and 0.99 (t, 3H, J=7.0 Hz, -CH$_2$-C**H**$_3$); ir ($CHCl_3$, cast) 2961 (OH), 1760 (ester C=O), 1734 (ester C=O), 1611 (ketone C=O), and 1466, 1457, 1443 cm^{-1} (Ar-**H**); hreims, m/z 338.13607 M$^+$ (calcd. for $C_{17}H_{22}O_7$ 338.13657), 278.11542 (56), 236.10486 (64), 193.04978 (100); ^{13}C-nmr (100.6 MHz, $CDCl_3$) δ 207.2 (p), 166.9 (p), 155.0 (p), 111.1 (p), 109.1(p), 103.4 (p), 96.3 (p), 90.9 (a), 62.8 (p), 55.7 (o), 46.6 (p), 22.7 (p), 21.0 (o), 20.9 (o), 17.8 (p), 13.9 (o).

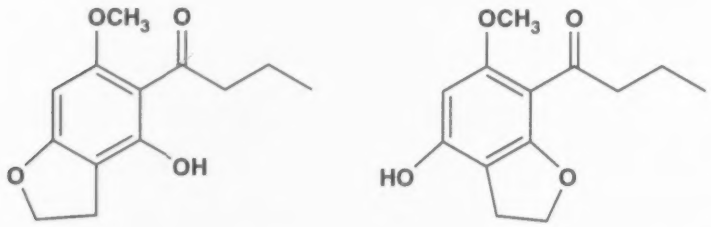
III. G. 2. Phomalone triacetate [2,4-diacetoxy-3-(2-acetoxyethyl)-6-methoxybutyrophenone (**30**)]



Triethylamine (0.3 mL, 0.22 mmol, 7 eq.), and acetic anhydride (0.3 mL, 0.3 mmol, 10 eq.) were added to a solution of phomalone (7.8 mg, 0.03 mmol) in methylene chloride (2 mL). After stirring the mixture for 2 h at room temperature, the solution was concentrated under reduce pressure and the residue was dissolved in chloroform (3 mL). Prep-TLC (10% ethyl acetate in methylene chloride) gave phomalone triacetate [2,4-diacetoxy-3-(2-acetoxyethyl)-6-methoxybutyrophenone] (R_f=0.35, 10.5 mg, 90%): ^{1}H-nmr (400 MHz, $CDCl_3$) δ 6.13 (s, 1H, Ar-**H**), 4.10 (t, 2H, J=7.0 Hz, -CH$_2$-C**H**$_2$-), 3.82 (s, 3H, -OC**H**$_3$), 2.79 (t, 2H, J=7.0 Hz, Ar-C**H**$_2$-), 2.72 (t, 2H, O=C-C**H**$_2$-), 2.40 (s, 3H, -O-C=O-C**H**$_3$), 2.27 (s, 3H, -O-C=O-C**H**$_3$), 2.05 (s, 3H, -O-C=O-C**H**$_3$), 1.66 (tq, 2H, J=J'=7.0 Hz, -CH$_2$-C**H**$_2$-CH$_3$), and 0.95 (t, 3H, J=7.0 Hz, -CH$_2$-C**H**$_3$); ir ($CHCl_3$, cast) 1772, 1739 (ester -O-C=O), 1699

(ketone C=O), and 1464, 1445 cm^{-1} (Ar-H); hreims, m/z 380.1468 M^+ (calcd. for $C_{19}H_{24}O_8$ 380.1461), 278.1154 (98), 236.1046 (39); ^{13}C-nmr (100.6 MHz, $CDCl_3$) δ 202.7 (p), 170.9 (p), 169.9 (p), 169.1 (p), 156.3 (p), 151.4 (p), 147.5 (p), 122.6 (p), 116.0 (p), 104.3 (a), 62.7 (p), 56.1 (o), 45.9 (p), 24.2 (p), 20.9 (o), 20.6 (o), 17.3 (p), 13.7 (o).

III. G.3. 4-Hydroxy-6-methoxy-5-(1'-oxobutyl)benzo[b]dihydrofuran (**31**) and 4-hydroxy-6-methoxy-7-(1'-oxobutyl)benzo[b]dihydrofuran (**32**)



III. G. 3.1. Using *p*-toluenesulfonic acid

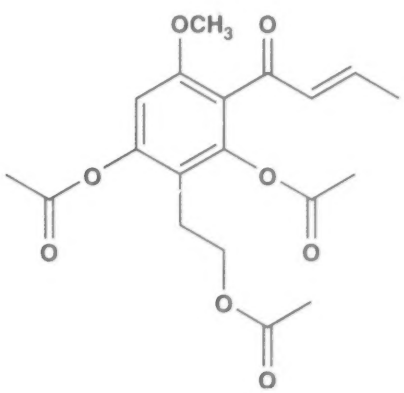
p-Toluenesulfonic acid monohydrate (5.3 mg, 0.028 mmol, 1 eq.) was added to a solution of phomalone (7.5 mg, 0.03 mmol) in benzene (5 mL). After stirring at room temperature for 1.5 h, another 2 eq. (10.6 mg, 0.06 mmol) of acid was added and the mixture was refluxed for 4.5 h with continuous removal of water (Dean-Stark). The solvent was then evaporated under reduce pressure and the residue was dissolved in chloroform (3 mL). Prep-TLC (10% ethyl acetate in methylene chloride) gave 4-hydroxy-6-methoxy-7-(1'-oxo–butyl)benzo[b]dihydrofuran and 4-hydroxy-6-methoxy-5-(1'-oxobutyl)-benzo[b]dihydrofuran in a ratio 2:1. 4-Hydroxy-6-methoxy-7-(1'-oxobutyl)-benzo[b]dihydrofuran: (R_f=0.60), 2.2 mg, 30%; ^1H-nmr (400 MHZ, $CDCl_3$) δ 9.50 (s, 1H, Ar-O**H**), 5.90 (s, 1H, Ar-**H**), 4.5 (t, 2H, J=7.0 Hz, -O-C**H**$_2$-CH_2-), 3.8 (s, 3H, -OC**H**$_3$), 3.2 (t, 2H, J=7.0 Hz, -CH_2-C**H**$_2$-Ar), 2.80 (t, 2H, J=7.0 Hz, O=C-C**H**$_2$-), 1.60 (qt, 2H, J=J'= 7.0 Hz, -CH_2-C**H**$_2$-CH_3), and 0.98 (t, 3H, J=7.0 Hz, -O-CH_2-C**H**$_3$); ir ($CHCl_3$, cast) 3250 (OH), 1650 (ketone C=O), 1450, 1497 cm^{-1} (Ar-H); hreims, m/z 236.1055 M^+ (calcd. for $C_{13}H_{16}O_4$ 236.1062), 193.0501 (100), 149.0241 (30).

4-Hydroxy-6-methoxy-5-(1'-oxobutyl)benzo[b]dihydrofuran: (R_f=0.80), 1.4 mg, 20%; ^{1}H-nmr (400 MHZ, $CDCl_3$) δ 14.50 (s, 1H, Ar-O**H**), 5.90 (s, 1H, Ar-**H**), 4.5 (t, 2H, J=7.0 Hz, -O-C**H**$_2$-CH$_2$-), 3.8 (s, 3H, -OC**H**$_3$), 3.2 (t, 2H, J=7.0 Hz, -CH$_2$-C**H**$_2$-Ar), 2.80 (t, 2H, J=7.0 Hz, O=C-C**H**$_2$-), 1.60 (qt, 2H, J=J'=7.0 Hz, -CH$_2$-C**H**$_2$-CH$_3$), and 0.98 (t, 3H, J=7.0 -O-CH$_2$-C**H**$_3$); ir ($CHCl_3$, cast) 3250 (OH), 1650 (ketone C=O), 1450, 1497 cm^{-1} (Ar-H); hreims, m/z 236.1055 M$^+$ (calcd. for $C_{13}H_{16}O_4$ 236.1062), 193.0501 (100), 149.0241 (30); ^{13}C-nmr (100.6 MHz, $CDCl_3$) δ 206.4 (p), 165.9(p), 164.3 (p), 162.7 (p), 142.6 (p), 132.9 (p), 92.4 (o), 62.9 (p), 55.9 (o), 46.6 (p), 26.4 (p), 18.9 (p), and 14.2 (o).

III. G. 3. 2. Using *p*-toluenesulfonyl chloride

p-Toluenesulfonyl chloride (45 mg, 0.24 mmol, 6 eq.) was added to a solution of phomalone (10 mg, 0.039 mmol) in benzene (15 mL). The mixture was refluxed for 4.5 h. The solvent was then evaporated under reduced pressure and the residue was dissolved in chloroform (3 mL). Prep-TLC (10% ethyl acetate in methylene chloride) gave 4-hydroxy-6-methoxy-7-(1'-oxobutyl)benzo[b]dihydrofuran (R_f=0.60, 5 mg, 50%), and 4-hydroxy-6-methoxy-5-(1'-oxobutyl)benzo[b]dihydrofuran (R_f=0.80, 2.5 mg, 25 %). Properties are described in the previous section.

III. G.4. (*E*)-2,4-acetoxy-3-(2-acetoxyethyl)-6-methoxy-2'-enbutyrophenone (**36**)



MM-liquid culture (2 L, 13 days shaking) were prepared according to the procedure described in section III. C. 7. 1. The floating HP-20 ion exchange resin was decanted from the broth. The remaining resin which was adhered to the mycelium was separated by vacuum filtration through Whatman paper No.1. The resin and the mixture resin-mycelium were separately transferred to a glass-filter (porosity D, ASTM 10-20 μm) and extracted with ethyl acetate (4·400 mL). The ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give a brown viscous oil (0.25 g). Flash chromatography (10% ethyl acetate in methylene chloride) gave an impure sample of (*E*)-2,4-dihydroxy-3-(2-hydroxyethyl)-6-methoxy-2'-en-butyrophenone (23 mg). Acetylation according to the procedure described in section II. G. 2. gave a brown oil (22 mg). Preparative TLC of this residue (10% ethyl acetate in methylene chloride) afforded (*E*)-2,4-acetoxy-3-(2-acetoxyethyl)-6-methoxy-2'-en-butyrophenone as a colorless liquid (2 mg): R_f=0.33, ^{1}H-nmr (400 MHz, $CDCl_3$) δ 6.64 (s,1H, Ar-**H**), 6.75 (dq, 1H, J=16.5 Hz, J'=7.0 Hz, O=C-CH=C**H**-), 6.34 (dq, 1H, J=16.5 Hz, J'=2.0 Hz, -C**H**=CH-CH_3), 4.11 (t, 3H, J=7.5 Hz, -CH_2-C**H$_2$**-OAc), 3.77 (s, 3H, -O-C**H$_3$**), 2.71 (t, 2H, J=7.5 Hz, Ar-C**H$_2$**-CH_2-), 2.38 (s, 3H, -O-C=O-C**H$_3$**), 2.23 (s, 3H, -O-C=O-C**H$_3$**), 2.03 (s, 3H, -O-C=O-C**H$_3$**), and 1.92 (dd, 1H, J=7.0 Hz, J'=2.0 Hz, =CH-C**H$_3$**); ir ($CHCl_3$, cast) 1771 (C=O ester), 1738 (C=O ester), 1660 (C=O ketone), 1617 (C=C), 1464, 1441 (Ar-**H**), and 968 cm^{-1} (-C**H**=C**H**-); ^{13}C-nnr ($CHCl_3$, 100.6 MHz) δ 202.7 (p), 170.9 (p), 169.1 (p), 168.9 (p), 156.3 (p), 151.3 (p), 147.6 (p), 146.9 (p), 132.5 (p), 120.9 (p), 115.9 (p), 104.3 (o), 62.6 (p), 56.0 (o), 23.9 (p), 21.0 (o), 20.9 (o), and 17.2 (o).

III. H. ^{13}C-LABELED-PHOMALONE

III. H. 1. Isolation of ^{13}C-labelled phomalone

MM-liquid cultures (2 L, 13 days shaking) were prepared according to the procedure described in section II. C. 7. 1. The floating HP-20 ion exchange resin was decanted from the broth. The remaining resin which adhered to the mycelium was separated by vacuum filtration through Whatman paper No.1. The resin and the resin-mycelium mixture were separately transferred to a

glass-filter (porosity D, ASTM 10-20 μm) and extracted with ethyl acetate (4·400 mL). The ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give a resin crude extract (548 mg, after feeding experiment with [1-^{13}C]-sodium acetate). Flash chromatography of this resin extract (10% ethyl acetate in methylene chloride) followed by HPMPLC (NP-Si60, 10% ethyl acetate in methylene chloride) and HPLC (Waters, μBondapak C-18, 7.8·300 mm, 10 μm; gradient: 40% water in methanol to 100% methanol) gave ^{13}C-labelled phomalone (6 mg). The same procedure was repeated with [2-^{13}C] and [1,2-$^{13}C_2$]-sodium acetate. Properties for phomalone are described in sections III. E. 1.

III. H. 2. Preparation of ^{13}C-labeled diacetate and triacetate derivatives

Triethylamine (0.2 mL, 0.15 mmol, 3.3 eq.), and acetic anhydride (0.2 ml, 0.2 mmol, 5 eq.) were added to a solution of ^{13}C-labelled phomalone (11 mg, 0.04 mmol) in methylene chloride (2 mL). After stirring the mixture for 1.5 h. at room temperature, the solution was concentrated under reduced pressure and the residue was dissolved in chloroform (3 mL). Prep-TLC (10% ethyl acetate in methylene chloride) gave ^{13}C-labelled phomalone diacetate (R_f=0.60, 6.8 mg) and ^{13}C-labelled phomalone triacetate (R_f=0.35, 7.6 mg) in a 1:1 ratio. The same procedure was used for ^{13}C-labelled phomalone that was obtained after feeding experiments with [2-^{13}C] and [1,2-$^{13}C_2$]-sodium acetate. Properties for phomalone diacetate and phomalone triacetate are described in sections III. G. 1 and III. G. 2 respectively.

II. I. BIOASSAY

II. I. 1. Filter paper in solid media

The inoculum was obtained from the growing edge of the colony of *P. tremulae* in MEA Petri dishes. After ten days of growing at room temperature, a plug (7 mm diameter) cut from this culture was placed in the Petri dish (2%MEA). The sample was sterilized by filtration through a 0.22 μm nylon membrane, and adsorbed on a filter paper (10 mm diameter). The

solvent was evaporated in a sterile fumehood for 3 h, and the filter paper was placed in the *P. tremulae* Petri dish. Three samples and the solvent (control) were evaluated for each MEA Petri dishes. This procedure was repeated for fractions showing the same R_f value on each chromatographic step. This bioassay showed positive or negative results. The bioassay was evaluated by the growth of *P. remulae* in the surronding of the filter paper. A positive results was obtained for an inhibition in the growing edge of a colony of *P. remulae* .

III. I. 2. Multiwell method

The sample was sterilized by filtration through a 0.22 μm nylon membrane. A 200 μL aliquot was applied directly to the well of the multiwell-plate (24-well, flat bottom, Falcon 3047) using a micropipette. The solvent was evaporated in a sterile fumehood for 3 h. The inoculum was obtained from the growing edge of a colony of *Phellinus tremulae* in MEA Petri dishes. After ten days of growth at room temperature, a plug (7 mm diameter) cut from this culture was placed in each well of the multiwell plate. Four replicates for each sample and the solvent (control) were evaluated in each multiwell plate. This procedure was repeated for fractions showing the same R_f value on each chromatographic step. A positive results was obtained for an inhibition in the radial growth of a colony of *P. tremulae* relative to control.

III. I. 2. Pure compounds

The sample was sterilized by filtration through a 0.22 μm nylon membrane. A 0.25 mL aliquot of a standard solution was applied directly to the well of the multiwell plate (6-well, flat bottom, Falcon 3046) using a micropipette. The solvent was evaporated in a sterile fumehood for 3 h. The inoculum was obtained from the growing-edge colony of *P. tremulae* in MEA Petri dishes. After ten days of growing at room temperature, a plug (7 mm diameter) cut from this culture was placed in each well of the multiwell plate. Three replicas for each sample and the solvent (control) were evaluated for each multiwell plate. This procedure was repeated for seven different concentrations: $4 \cdot 10^{-3}$, $2 \cdot 10^{-3}$, $1 \cdot 10^{-3}$, $4 \cdot 10^{-4}$, $2 \cdot 10^{-4}$, $1 \cdot 10^{-4}$, and $4 \cdot 10^{-5}$ mol·L^{-1}. The bioassay was

evaluated by the amount of growth of *P. tremulae*, relative to control, in each well. The radial growth was measured eight, fourteen and thirty days after inoculation by dividing by two the mean of two diameter readings of each well taken at right angle to each other.

REFERENCES

1. Y. Hiratsuka, D. Gibbard, O. Bakowsky, and G. Maier. "Classification and measurement of aspen decay and stain in Alberta," Can. For. Serv.; North. For. Res. Cent.; Edmonton, Alberta, 1990. Inf. Rep. NOR-X-314.

2. G. Thomas. *Abstracts of paper,* The future for poplar utilization in Alberta, Canada. Prod. Ind. Wood Prod. Semin., Edmonton; Can. Dep. Ind.; Wood Prod. Branch Ottawa:Ontario , 1966; Abstract number 49.

3. J. Lindsey, and R. Gilbertson. *Basidiomycetes that decay aspen in North America*; R Gantner Verlag: Lehre, 1978, p, 406.

4. J. Ginns. "Compendium of plant disease and decay fungi in Canada. 1960-1980," Res. Branch. Agric. Canada, 1986. Publication No. 1813. p, 416 .

5. H. Good, and J. Nelson. *Can. J. Bot.* **1962**, *40*, 615.

6. G. Laflamme, and M. Lortie. *Can. J. For. Res.* **1973**, *3*, 155.

7. A. Shigo. *Int. Rev. For. Res.* **1967**, *2*, 237.

8. J. Bashan. *Can. J. Bot.* **1958**, *36*, 491.

9. A. Cenry. *Acta Universitatis Agriculturae (Brno) Ser. C* **1972**, *41*, 131.

10. H. Schrenk, and P. Spaulding. "Disease of decidious forest trees," U. S. D. A.; Bureau of Plant Ind., 1909. Bull. 149.

11. C. Wikstrom. *Eur. J. For. Path.* **1976**, *6*, 321.

12. C. Wikstrom, and T. Unestam. *Eur. J. For. Path.* **1976**, *6*, 291.

13. G. Thomas, D. Etheridge, and D. Paul. *Can. J. Bot.* **1960**, *38*, 459.

14. J. T. Bashan. "The effects of decay on the production of trembling aspen pulpwood in the Upper Pic region of Ontario," Can. Dep. Agric., For. Biol. Div.; Ottawa, 1960. Publ. 1060.

15. L. Hutchison, and Y. Hiratsuka. Presented at The Mycological Society America/American Phytopathological Society joint meeting, Portland; August 1992; paper number 450.

16. L. Hutchison, P. Chakravarty, and Y. Hiratsuka. Presented at The Canadian Phytopathological Society Meeting, Charlottetow, Prince Edward Island; July 1992; paper number 710.

17. L. Hutchison, P. Chakravarty, and Y. Hiratsuka. *Can. J. Bot.*, in press.

18. E. Peterson, and N. Peterson. "Ecology, management, and use of Aspen and Balsam poplar in the Prairie Provinces, Canada," Can. For. Serv., North. For. Res. Cent.; Edmonton, 1992. Special report 1.

19. *Poplars and willows in wood production and land use*; FAO, Ed.; Food and Agricuture Organization of the Unites Nations; Forestry Series: Rome, 1979; Vol. 10, p, 12.

20. T. Braysaw. "Native poplars of southern Alberta and their hybrids," Can. Dep. For.; For. Res. Branch, Ottawa, 1965. Dep. For. Publ. 1109.

21. H. Scoggan. "The flora of Canada," Natl. Mus. Can.; Ottawa, Ontario, 1978. Publ. Bot. 7.

22. R. Taylor, and B. MacBryde. "Vascular plants of British Columbia: a descriptive resource inventory," University of British Columbia, 1977. Univ. B. C. Bot. Gard. Tech. Bull. 4.

23. D. Dickmann, and K. Stuart. *The cuture of poplars in Eastern North America*; Michigan State University; Department of Forestry: Michigan, 1983, p, 12.

24. G. Schier. "Vegetative propagation of Rocky Montain aspen," U. S. For. Serv., 1978. General technical report INT-44.

25. G. Schier. "Rooting stem cuttings from aspen seedlings," U. S. For. Serv., 1980. Research note INT -282.

26. G. Schier. "Physiological research on adventitious shoot development in aspen roots," U. S. For. Serv., 1981. General technical report INT-107.

27. D. Perala. "Clone expansion and copetition between quaking and bigfoot aspen suckers after clearcutting," U. S. For. Serv., 1981. Research paper NC-201.

28. E. Little. " Atlas of United States trees. Vol. 1. Conifers and important harwoods," U. S. Dep. Agric.; For. Ser.; Washington, D. C., 1971. Misc. Publ. No. 1146.

29. J. Maini. In *Growth and utilization of poplar in Canada*; J. Maini, and J. Cayford, Ed.; Can. Dep. For. Rural Devel.; For. Branch: Ottawa, 1968; p, 20.

30. J. Fitzpatrick, and J. Stewart. In *Growth and utilization of poplar in Canada*; J. Maini, and J. Cayford, Ed.; Can. Dep. For. Rural Dev.; For. Branch: Ottawa, 1968; p, 214.

31. K. Hunt, J. Bashan, and J. Kemperman. *Can. J. For. Res.* **1978**, *8*, 181.

32. D. Breck. Presented at The Aspen Quality Workshop Proceedings, Edmonton; Can. For. Serv.; North. For. Center, Edmonton , February 1987; Abstract 6.

33. Y. Hiratsuka, and A. Loman. "Decay of aspen and balsam poplar in Alberta," Environ. Can.; Can. For. Serv.; North. For. Res. Cent., Edmonton, 1984. Inf. Rep. NOR-X-262.

34. W. Ondro. "Utilization and market potential of poplars in Alberta," Can. For. Serv.; North. For. Cent.; Edmonton, Alberta, 1989. Rep. NOR-X-305. p, 64 .

35. A. L. Shigo. *Plant. Dis. Rept.* **1963**, *47*, 820.

36. A. Shigo. "Organism interactions in decay and discoloration in beech, brich and maple," U. S. D. A.; For. Serv., 1965. Res. Pap. NE-43. p, 1 .

37. T. Hinds. In *Aspen: ecology and management in the Western United States*; N. Debyle, and R. Winokur, Ed.; U.S.D.A. For. Service: Washington, D.C., 1985; p, 106.

38. P. Crane. M.Sc. Thesis, University of Alberta, 1993.

39. P. Crane. Presented at The Plant Pathology Society of Alberta, Olds, Alberta; November 1992; paper 22.

40. K. Domsch, W. Gams, and T. Anderson. *Compendium of soil fungi*; Academic Press: New York, 1980; Vol. 1, p, 632.

41. I. Bick, and C. Chee. *Biochem. J.* **1966**, *98*, 112.

42. K. Schofield, and D. Wright. *J. Chem. Soc.* **1965**, 6642.

43. D. Wright, and K. Schofield. *Nature* **1960**, *188*, 233.

44. A. Birch, D. Butler, and R. Richards. *Nature* **1961**, *190*, 441.

45. A. Closse, R. Mauli, and H. Sigg. *Helv. Chim. Acta* **1966**, *49*, 204.

46. S. Sakamura, J. Niki, Y. Obata, R. Sakaki, and T. Matsumoto. *Agric. Biol. Chem.* **1969**, *33*, 698.

47. M. Sequin-Fray, and C. Tamm. *Helv. Chim. Acta* **1971**, *54*, 851.

48. R. Evans, G. Ellstad, and M. Kunstmann. *Tetrahedron Letters* **1969**, 1791.

49. G. McLaughlin, G. Sim, J. Kiechel, and C. Tamm. *Chem. Comn.* **1970**, 1398.

50. W. Rothweiler, and C. Tamm. *Experientia* **1966**, *22*, 750.

51. W. Rothweiler, and C. Tamm. *Helv. Chim. Acta* **1970**, *53*, 696.

52. D. Aldridge, J. Armstrong, R. Speake, and W. Turner. *J. Chem. Soc., (C)* **1967**, 1667.

53. D. Aldridge, J. Armstrong, R. Speake, and W. Turner. *Chem. Comm.* **1967**, 26.

54. W. McGahren, G. Ellstand, G. Morton, and M. Kunstman. *J. Org. Chem.* **1972**, *37*, 1636.

55. C. Takahashi, S. Sekita, K. Yoshihira, S. Natori, S. Udagawa, H. Kurata, M. Enamoto, K. Ohtsubo, M. Umeda, and M. Saito. *Chem. Pharm. Bull.* **1973**, *21*, 2286.

56. J. Deacon. In *Basic microbiology*; J. Wilkinson, Ed.; John Wiley & Sons: New York, 1980; Vol. 7; p, 216.

57. C. Leach. *Can. J. Bot.* **1962**, *40*, 1577.

58. C. Leach. *Can. J. Bot.* **1962**, *40*, 151.

59. D. Williams, and I. Fleming. *Spectroscopic methods in organic chemistry;* Fourth ed.; MacGraw-Hill Book Company: London, 1989, p, 114.

60. T. Simpson, I. Chandler, and C. McIntyre. *J. Chem. Soc. Perkin Trans.I* **1992**, 2273.

61. A. Abdel-Sayed, and L. Bauer. *Tetrahedron Lett.* **1986**, *27*, 1003.

62. A. Bax. *J. Magn. Reson.* **1984**, *57*, 314.

63. A. Bax, J. Ferretti, N. Nashed, and D. Jerima. *J. Org. Chem.* **1985**, *50*, 3029.

64. Z. Fomum, J. Atafor, and J. Mbafor. *Tetrahedron Lett.* **1983**, *24*, 4127.

65. T. Wu, H. Furukawa, C. Kuoh, and K. Hus. *J. Chem. Soc., Perkin Trans. I* **1983**, 1681.

66. R. Durley, J. MacMillan, and T. Simpson. *J. Chem. Soc., Perkin Trans. I* **1975**, 163.

67. L. Bellamy. *The Infra-Red Spectra of Complex Molecules*; Methuen: London, 1858.

68. R. Rasmussen, D. Tunniclif, and R. Brattain. *J. Am Chem. Soc.* **1949**, *71*, 1068.

69. M. Kobayashi, Y. Terui, K. Tori, and N. Tsuji. *Tetrahedron Lett.* **1976**, *8*, 619.

70. T. Simpson. *Chem. Soc. Rev.* **1975**, *4*, 497.

71. E. Bardshiri, and T. Simpson. *J. Chem. Soc., Chem. Commun.* **1981**, 195.

72. T. Simpson, I. Chandler, and C. McIntyre. *J. Chem. Soc. Perkin Trans. I* **1992**, 2285.

73. D. Rabenstein, and T. Nakashima. *Anal. Chem.* **1979**, *51*, 1465 A.

74. S. Patt, and J. Shoolery. *J. Mag. Res.* **1982**, *46*, 535.